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# GROWTH

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## A JOURNAL FOR STUDIES OF DEVELOPMENT AND INCREASE

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# STUDIES ON THE GROWTH-PROMOTING FACTOR OF ADULT TISSUE EXTRACTS

## V. THE EFFECT OF DIALYSIS*

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(Received for publication January 3, 1948)

The study reported in the present paper deals with the effect of dialysis on the cell growth promoting activity of adult tissue extracts.

The experiments were performed with third-passage cultures of fibroblasts originating from the hearts of eight-days old chick embryos. The methods of cultivation and of growth measurement were the same as those described in the first paper of this series (Doljanski and Doljanski, 1944).

### EXPERIMENTAL

The dialysis experiments were carried out with:

(A) Extract of fresh adult chicken hearts (Hoffman and Doljanski, 1939).

(B) Extract of acetone-desiccated adult chicken hearts (Margoliash, 1947).

### (A) EXPERIMENTS WITH EXTRACT OF FRESH ADULT CHICKEN HEARTS

1. *Procedure.*

Extracts were prepared from fresh adult chicken hearts under sterile conditions according to a previously described procedure (Hoffman and Doljanski, 1939). The dialysis flasks and tubing were autoclaved. The Tyrode's solution was sterilized by autoclaving separately the $NaHCO_3$ and the rest of the salts, and mixing the cooled solutions. Both for dialysis and testing, the heart extract was used in a dilution of 33%, since in order to observe relatively small differ-

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ences in stimulating potency it is obviously necessary to work with concentrations of tissue extracts which do not contain an excess of stimulating substances.

Dialyzed extract was obtained by dialyzing 12 mls. of diluted heart extract in washed Dupont cellophane tubing against one liter of Tyrode's solution. The outer solution was changed 3 times over a period of 4 days; the apparatus was maintained at +4° C. Dialysate of the fresh heart extract was obtained by dialyzing 3 mls. of Tyrode's solution against 12 to 14 mls. of concentrated fresh heart extract for 4 days in the refrigerator under sterile conditions. In order to combat occasional bacterial contamination the original heart extract, the dialyzed heart extract, and the dialysate were heated in a water bath at 60° C. for 30 minutes before being added to the cultures. This procedure has previously been shown to have no effect on the growth activating potency of heart extracts (Doljanski and Doljanski, 1944).

After centrifuging off the precipitate formed on heating the original and dialyzed heart extracts, the supernatant fluid of the original heart extract always appeared slightly opalescent, whereas in the case of the dialyzed heart extract it was entirely clear. The dialyzed extract had a slightly lighter reddish color than the undialyzed one. The dialysate usually appeared colorless, but on several occasions it was distinctly yellowish. The dialysate always gave a positive ninhydrin test.

2. *Results.*

(a) Reduction of the cell growth promoting activity of fresh heart extract following dialysis.

In this experiment the growth promoting power of a dialyzed 33% heart extract was compared with that of an undialyzed extract of the same concentration. The results summarized in Table 1 show that the dialysis caused a substantial reduction in the cell growth promoting activity of the heart extract.

(b) Cell growth promoting activity of the dialysate of fresh heart extract.

In this experiment the effect of the dialysate of fresh heart extract on cell growth in vitro was compared with that of Tyrode's solution. The results, summarized in Table 2, show that the mean size of the

TABLE 1

I THE AREA IN MM² OF 6 DAYS OLD FIBROBLAST COLONIES (SISTER HALVES) GROWING IN THE PRESENCE OF 33% FRESH HEART EXTRACT, UNDIALYZED (A) AND DIALYZED FOR 4 DAYS (B).

| No. of cultures | | A | B | No. of cultures | | A | B |
|---|---|---|---|---|---|---|---|
| 12797/98 | a | 75 | 42 | 12959/60 | a | 79 | 34 |
| | b | 75 | 48 | | b | 43 | 36 |
| 12799/00 | a | 64 | 53 | | c | 64 | 33 |
| | b | 42 | 22 | 12961/62 | a | 53 | 28 |
| | c | 58 | 32 | | b | 87 | 31 |
| 12801/02 | a | 44 | 44 | | c | 59 | 39 |
| | b | 44 | 33 | 12963/64 | a | 87 | 50 |
| | c | 60 | 53 | | b | 71 | 40 |
| 12803/04 | a | 63 | 28 | | c | 68 | 41 |
| | b | 42 | 36 | 12965/66 | a | 96 | 24 |
| | c | 59 | 32 | | b | 62 | 40 |
| 12805/06 | a | 48 | 42 | | c | 71 | 30 |
| | b | 37 | 32 | 12967/68 | a | 68 | 30 |
| | c | 57 | 45 | | b | 65 | 42 |
| 12807/08 | a | 46 | 31 | | c | 52 | 26 |
| | b | 58 | 37 | 12971/72 | a | 62 | 42 |
| | c | 69 | 46 | | b | 36 | 32 |
| 12819/20 | a | 41 | 20 | | c | 49 | 24 |
| | b | 39 | 22 | 12979/80 | a | 52 | 29 |
| | c | 58 | 39 | | b | 54 | 28 |
| 12821/22 | a | 39 | 16 | | c | 68 | 30 |
| | b | 56 | 18 | 12981/82 | a | 52 | 49 |
| | c | 27 | 8 | | b | 41 | 48 |
| 12823/24 | a | 53 | 36 | | c | 41 | 43 |
| | b | 35 | 20 | 12983/84 | a | 83 | 36 |
| | c | 42 | 21 | | b | 87 | 43 |
| 12825/26 | a | 62 | 25 | 12989/90 | a | 41 | 36 |
| | b | 51 | 31 | | b | 39 | 24 |
| | c | 53 | 24 | | c | 31 | 34 |
| 12827/28 | a | 46 | 24 | 13060/61 | a | 44 | 15 |
| | b | 53 | 29 | | b | 81 | 20 |
| | c | 31 | 17 | 13062/63 | a | 77 | 31 |
| 12829/30 | a | 41 | 17 | | b | 44 | 27 |
| | b | 50 | 11 | 13064/65 | a | 49 | 22 |
| | c | 50 | 20 | | b | 44 | 18 |
| 12833/34 | a | 34 | 14 | 13066/67 | a | 33 | 27 |
| | b | 81 | 38 | | b | 30 | 23 |
| | c | 47 | 22 | 13068/69 | a | 64 | 19 |
| 12835/36 | a | 54 | 18 | | b | 36 | 21 |
| | b | 29 | 23 | | c | 28 | 19 |
| 12937/38 | a | 31 | 26 | 13072/73 | a | 107 | 31 |
| | b | 33 | 26 | | b | 56 | 35 |
| | c | 42 | 22 | 13076/77 | a | 44 | 22 |
| 12939/40 | a | 55 | 16 | | b | 48 | 29 |
| | b | 30 | 15 | 13078/79 | a | 48 | 28 |
| 12945/46 | a | 46 | 18 | 13381/82 | a | 48 | 23 |
| | b | 31 | 30 | 13383/84 | a | 43 | 15 |
| | c | 50 | 34 | | b | 32 | 14 |
| 12949/50 | a | 41 | 29 | 14725/26 | a | 38 | 16 |
| | b | 22 | 24 | | b | 50 | 20 |
| | c | 40 | 25 | 14727/28 | a | 36 | 27 |
| 12953/54 | a | 66 | 34 | | b | 44 | 33 |
| | b | 72 | 29 | 14893/94 | a | 60 | 20 |
| | c | 73 | 39 | | b | 58 | 26 |
| 12955/56 | a | 50 | 29 | | | | |
| | b | 53 | 26 | | | | |
| | c | 64 | 35 | Average | | 52.4 | 29.1 |

cultures in the dialysate was slightly greater than that of the controls in the protective medium, but this difference is obviously too small to be significant.

TABLE 2

I. The Area in $mm^2$ of 6 Days Old Fibroblast Colonies (Sister Halves) Growing in the Presence of Tyrode's Solution (A) and of the Dialysate of Fresh Heart Extract (B).

| No. of cultures | | A | B | No. of cultures | | A | B |
|---|---|---|---|---|---|---|---|
| 12851/52 | a | 14 | 13 | 14643/44 | a | 18 | 17 |
| | b | 27 | 26 | | b | 19 | 19 |
| | c | 13 | 22 | 14711/12 | a | 25 | 22 |
| 12853/54 | a | 12 | 14 | | b | 23 | 17 |
| | b | 10 | 8 | 14713/14 | a | 8 | 10 |
| | c | 10 | 9 | | b | 11 | 7 |
| 12855/56 | a | 12 | 8 | 14715/16 | a | 12 | 10 |
| | b | 11 | 12 | | b | 14 | 10 |
| | c | 10 | 10 | | c | 14 | 12 |
| 12857/58 | a | 10 | 12 | 14789/90 | a | 11 | 16 |
| | b | 11 | 8 | | b | 12 | 16 |
| 12861/62 | a | 9 | 10 | 14793/94 | a | 8 | 13 |
| | b | 9 | 13 | | b | 11 | 9 |
| 12863/64 | a | 7 | 6 | 14805/06 | a | 18 | 29 |
| | b | 9 | 10 | | b | 15 | 21 |
| | c | 12 | 11 | | c | 13 | 15 |
| 12865/66 | a | 11 | 21 | 14859/60 | a | 16 | 13 |
| | b | 15 | 12 | | b | 11 | 17 |
| | c | 9 | 12 | 14861/62 | a | 13 | 16 |
| 12867/68 | a | 10 | 10 | | b | 12 | 13 |
| | b | 9 | 8 | | c | 14 | 8 |
| | c | 8 | 12 | 15051/52 | a | 13 | 12 |
| 12985/86 | a | 14 | 11 | | b | 11 | 12 |
| | b | 11 | 11 | 15063/64 | a | 14 | 12 |
| | c | 8 | 9 | | b | 10 | 12 |
| 13365/66 | a | 17 | 13 | 15065/66 | a | 13 | 15 |
| | b | 13 | 14 | | b | 16 | 16 |
| 13367/68 | a | 11 | 15 | 15089/90 | a | 11 | 13 |
| | b | 15 | 17 | | b | 11 | 17 |
| 13371/72 | a | 12 | 12 | 15109/10 | a | 14 | 11 |
| | b | 10 | 8 | | b | 12 | 11 |
| | c | 15 | 15 | | c | 9 | 10 |
| 13839/40 | a | 18 | 23 | 15111/12 | a | 11 | 12 |
| | b | 13 | 15 | | b | 15 | 20 |
| 13915/16 | a | 23 | 19 | | c | 9 | 10 |
| 13931/32 | a | 18 | 17 | 15131/32 | a | 10 | 13 |
| | b | 16 | 17 | | b | 14 | 14 |
| 14279/80 | a | 23 | 27 | 15133/34 | a | 12 | 15 |
| | b | 29 | 26 | | b | 14 | 13 |
| | c | 17 | 26 | | | | |
| 14289/90 | a | 13 | 15 | | | | |
| | b | 11 | 25 | Average | | 13.2 | 14.2 |

## (B) Experiments with Extracts from Acetone-Desiccated Adult Chicken Hearts

1. *Procedure.*

Extracts were prepared from acetone-desiccated chicken hearts according to a method previously described (Margoliash, 1947) and sterilized by passing through a Seitz filter pad. Usually about 350 mls. of extract were filtered at one time. Previous experiments have shown that when more than 50 mls. of extract from desiccated material are used the filtration causes no appreciable loss of growth promoting activity.

Dialyzed extract was obtained by dialyzing 25 mls. of the extract from acetone-desiccated chicken hearts in washed Dupont cellophane tubing against one liter of Tyrode's solution under sterile conditions. The outer solution was changed 3 times over periods of 3, 7 or 15 days and the apparatus was maintained at $+4^{\circ}$ C. The volume of the dialyzed extract in the cellophane tube was checked at the end of the period of dialysis. It was always found to be unchanged. The extracts from acetone-desiccated chicken hearts were never heated as they proved to be always sterile at the end of the period of dialysis.

Dialysate of the heart extract was obtained by dialyzing 20 mls. of Tyrode's solution against 250 mls. of heart extract for 3, 7 or 15 days in the refrigerator.

The dialyzed heart extract was slightly paler than the reddish original extract from acetone-desiccated chicken hearts, and there was no precipitate or opalescence formed. The dialysate was invariably yellow, giving a strongly positive ninhydrin test.

The dialyzed extract was always used in the original concentration. Its activity was defined by comparison with 50% and 25% dilutions of the undialyzed extract from acetone-desiccated material. In order to find out whether any activity was retained, the dialyzed extract was also compared to Tyrode's solution.

2. *Results.*

(a) Reduction of the cell growth promoting activity of extract of acetone-desiccated chicken hearts following dialysis.

The results obtained by the dialysis of the extract from acetone-desiccated hearts, after 3, 7, and 15 days are summarized in Tables 3, 4 and 5 respectively. The growth of cultures in the presence of the

undiluted dialyzed extract was compared with that in Tyrode's solution, undialyzed 25% extract, and in the case of the 3 days dialyzed extract, also with that in undialyzed 50% extract.

These tables show that after 3 days of dialysis the activity of the extract was reduced to slightly more than 25% of its original strength.

TABLE 3

I. The Area in $mm^2$ of 6 Days Old Fibroblast Colonies (Sister Halves) Growing in the Presence of 50% Extract from Acetone Dessicated Hearts (A), and of Undiluted Extract from Acetone Dessicated Hearts Dialyzed for 3 Days (B).

| No. of cultures | | A | B | No. of cultures | | A | B |
|---|---|---|---|---|---|---|---|
| 14743/44 | a | 75 | 58 | 14781/82 | a | 72 | 39 |
| | b | 65 | 45 | | b | 88 | 45 |
| 14745/46 | a | 54 | 36 | 15241/42 | a | 72 | 48 |
| | b | 31 | 22 | | b | 94 | 45 |
| 14747/48 | a | 48 | 28 | 15243/44 | a | 88 | 54 |
| | b | 42 | 28 | 15245/46 | a | 58 | 31 |
| 14779/80 | a | 61 | 36 | | | | |
| | b | 105 | 55 | Average | | 68.1 | 40.7 |

II. The Area in $mm^2$ of 6 Days Old Fibroblast Colonies (Sister Halves) Growing in the Presence of 25% Extract from Acetone Desiccated Hearts (A), and of Undiluted Extract from Acetone Dessicated Hearts Dialyzed for 3 Days (B).

| No. of cultures | | A | B | No. of cultures | | A | B |
|---|---|---|---|---|---|---|---|
| 14749/50 | a | 38 | 28 | 15253/54 | a | 40 | 58 |
| | b | 38 | 34 | | b | 48 | 61 |
| 14751/52 | a | 30 | 42 | 15255/56 | a | 72 | 67 |
| | b | 50 | 62 | | b | 54 | 56 |
| 14753/54 | a | 43 | 52 | 15257/58 | a | 59 | 70 |
| 14783/84 | a | 45 | 48 | | b | 56 | 54 |
| | b | 42 | 36 | 15259/60 | a | 43 | 48 |
| 15247/48 | a | 54 | 48 | | b | 46 | 48 |
| | b | 54 | 36 | | | | |
| 15251/52 | a | 50 | 59 | | | | |
| | b | 42 | 43 | Average | | 47.6 | 50.0 |

III. The Area in $mm^2$ of 6 Days Old Fibroblast Colonies (Sister Halves) Growing in the Presence of Tyrode's Solution (A), and of Undiluted Extract from Acetone Desiccated Hearts Dialyzed for 3 Days (B).

| No. of cultures | | A | B | No. of cultures | | A | B |
|---|---|---|---|---|---|---|---|
| 14755/56 | a | 14 | 36 | 15265/66 | a | 14 | 50 |
| | b | 14 | 42 | | b | 14 | 37 |
| 14757/58 | a | 13 | 28 | 15267/68 | a | 11 | 40 |
| | b | 10 | 26 | | b | 9 | 47 |
| 15261/62 | a | 10 | 39 | | | | |
| 15263/64 | a | 14 | 35 | | | | |
| | b | 9 | 34 | Average | | 12.0 | 37.6 |

After both 7 and 15 days of dialysis the activity of the dialyzed extract was about the same and was reduced to distinctly less than 25% of its original strength. This is apparently the greatest reduction that can be obtained by dialysis of heart extract, since after 7 days of dialysis there was no further loss.

TABLE 4

I. THE AREA IN MM² OF 6 DAYS OLD FIBROBLAST COLONIES (SISTER HALVES) GROWING IN THE PRESENCE OF 25% EXTRACT FROM ACETONE DESICCATED HEARTS (A), AND OF UNDILUTED EXTRACT FROM ACETONE DESSICATED HEARTS DIALYZED FOR 7 DAYS (B).

| No. of cultures | | A | B | No. of cultures | | A | B |
|---|---|---|---|---|---|---|---|
| 14811/12 | a | 54 | 45 | 15225/26 | a | 54 | 38 |
| | b | 53 | 25 | | b | 54 | 36 |
| 14813/14 | a | 51 | 32 | 15227/28 | a | 53 | 36 |
| | b | 38 | 30 | | b | 72 | 44 |
| 14871/72 | a | 96 | 45 | 15229/30 | a | 51 | 30 |
| | b | 55 | 33 | | b | 68 | 40 |
| 14873/74 | a | 54 | 24 | 15231/32 | a | 49 | 30 |
| | b | 107 | 88 | | b | 89 | 45 |
| 14875/76 | a | 50 | 33 | 15233/34 | a | 58 | 38 |
| | b | 58 | 35 | | b | 58 | 32 |
| 14877/78 | a | 100 | 54 | | | | |
| | b | 48 | 31 | | | | |
| 15223/24 | a | 39 | 31 | Average | | 61.3 | 38.0 |

II. THE AREA IN MM² OF 6 DAYS OLD FIBROBLAST COLONIES (SISTER HALVES) GROWING IN THE PRESENCE OF TYRODE'S SOLUTION (A), AND OF UNDILUTED EXTRACT FROM ACETONE DESICCATED HEARTS DIALYZED FOR 7 DAYS (B).

| No. of cultures | | A | B | No. of cultures | | A | B |
|---|---|---|---|---|---|---|---|
| 14849/50 | a | 31 | 50 | 14917/18 | a | 12 | 45 |
| | b | 28 | 58 | | b | 11 | 43 |
| 14851/52 | a | 32 | 68 | 15237/38 | a | 11 | 45 |
| 14853/54 | a | 28 | 61 | | b | 18 | 37 |
| | b | 37 | 64 | 15239/40 | a | 18 | 34 |
| 14913/14 | a | 9 | 37 | | b | 22 | 42 |
| | b | 13 | 45 | | | | |
| | c | 14 | 48 | | | | |
| 14915/16 | a | 11 | 31 | | | | |
| | b | 13 | 36 | Average | | 19.2 | 46.5 |

(b) Presence of some growth promoting activity in the dialysate of extract from acetone-desiccated chicken hearts.

The growth of cultures in the presence of the dialysate from acetone-desiccated hearts after 3, 7, and 15 days of dialysis was compared with (a) that in Tyrode's solution and (b) that in 25% extract from acetone-desiccated hearts. The results summarized in

TABLE 5

I. The Area in $mm^2$ of 6 Days Old Fibroblast Colonies (Sister Halves) Growing in the Presence of 25% Extract from Acetone Desiccated Hearts (A), and of Undiluted Extract from Acetone Dessicated Hearts Dialyzed for 15 Days (B).

| No. of cultures | | A | B | No. of cultures | | A | B |
|---|---|---|---|---|---|---|---|
| 15183/84 | a | 68 | 36 | 15191/92 | a | 64 | 42 |
| | b | 72 | 48 | | b | 53 | 36 |
| 15185/86 | a | 45 | 28 | 15193/94 | a | 48 | 30 |
| 15187/88 | a | 61 | 36 | | b | 36 | 25 |
| | b | 60 | 45 | | | | |
| 15189/90 | a | 54 | 43 | | | | |
| | b | 54 | 33 | Average | | 55.8 | 36.5 |

II. The Area in $mm^2$ of 6 Days Old Fibroblast Colonies (Sister Halves) Growing in the Presence of Tyrode's Solution (A), and of Undiluted Extract from Acetone Desiccated Hearts Dialyzed for 15 Days (B).

| No. of cultures | | A | B | No. of cultures | | A | B |
|---|---|---|---|---|---|---|---|
| 15195/96 | a | 21 | 46 | 15201/02 | a | 10 | 40 |
| | b | 13 | 26 | | b | 9 | 46 |
| 15197/98 | a | 20 | 45 | 15203/04 | a | 14 | 31 |
| | b | 26 | 33 | | b | 10 | 38 |
| 15199/00 | a | 14 | 47 | 15205/06 | a | 17 | 38 |
| | b | 14 | 40 | | b | 13 | 33 |
| | | | | Average | | 15.1 | 38.6 |

Table 6 indicate that the cultures grown in the presence of the dialysate showed a somewhat greater area than the cultures grown in the presence of Tyrode's solution. The area of the cultures grown in the dialysate was, however, considerably less than that of cultures grown in 25% heart extract.

It should be made clear that the results of this experiment with dialysate from acetone-desiccated hearts are not in conformity with those obtained with the dialysate of fresh heart extract, which as shown above had no appreciable effect on the growth of cells in vitro. This may have been caused by differences in the experimental conditions, which in the case of the dried material extracts were far more favorable to the securing of a thorough dialysis than in the case of the fresh extracts. Indeed, much larger quantities of extract from acetone-desiccated chicken hearts were used than of fresh extract, so that the dialysis surface was much larger, and the final dilution much smaller for the dried material extracts than for the fresh extracts. Moreover, adequate dialysis may have been prevented

TABLE 6

I THE AREA IN MM² OF 6 DAYS OLD FIBROBLAST COLONIES (SISTER HALVES) GROWING IN THE PRESENCE OF TYRODE'S SOLUTION (A), AND OF THE DIALYSATE OF EXTRACT FROM ACETONE DESICCATED HEARTS (B).

| No. of cultures | | A | B | No. of cultures | | A | B |
|---|---|---|---|---|---|---|---|
| *(after 3 days of dialysis)* | | | | *(after 15 days of dialysis)* | | | |
| 14763/64 | a | 14 | 26 | 14625/26 | a | 26 | 45 |
| | b | 11 | 30 | | b | 16 | 36 |
| 14767/68 | a | 14 | 20 | 14627/28 | a | 18 | 42 |
| | b | 18 | 35 | | b | 18 | 38 |
| 15171/72 | a | 16 | 21 | 14665/66 | a | 10 | 28 |
| | b | 16 | 16 | | b | 8 | 23 |
| 15173/74 | a | 9 | 24 | 14683/84 | a | 6 | 14 |
| | b | 9 | 23 | | b | 6 | 16 |
| *(after 7 days of dialysis)* | | | | 14685/86 | a | 5 | 25 |
| 14833/34 | a | 16 | 24 | | b | 6 | 27 |
| | b | 20 | 26 | | | | |
| 14835/36 | a | 10 | 30 | | | | |
| | b | 16 | 39 | | | | |
| 14883/84 | a | 18 | 28 | | | | |
| | b | 14 | 36 | | | | |
| 14907/08 | a | 13 | 26 | | | | |
| | b | 12 | 27 | | | | |
| 14909/10 | a | 12 | 28 | | | | |
| 14911/12 | a | 16 | 30 | Average | | 13.3 | 27.9 |

II. THE AREA IN MM² OF 6 DAYS OLD FIBROBLAST COLONIES (SISTER HALVES) GROWING IN THE PRESENCE OF 25% EXTRACT FROM ACETONE DESICCATED HEARTS (A), AND OF THE DIALYSATE OF EXTRACT FROM ACETONE DESICCATED HEARTS (B).

| No. of cultures | | A | B | No. of cultures | | A | B |
|---|---|---|---|---|---|---|---|
| *(after 3 days of dialysis)* | | | | *(after 15 days of dialysis)* | | | |
| 14759/60 | a | 35 | 25 | 14629/30 | a | 54 | 27 |
| 15179/80 | a | 40 | 16 | 14631/32 | a | 40 | 31 |
| 15181/82 | a | 45 | 25 | 14633/34 | a | 64 | 31 |
| *(after 7 days of dialysis)* | | | | | b | 45 | 29 |
| 14829/30 | a | 71 | 39 | 14635/36 | a | 38 | 23 |
| | b | 68 | 48 | 14665/66 | a | 80 | 32 |
| 14831/32 | a | 66 | 27 | 14667/68 | a | 42 | 23 |
| | | | | | b | 27 | 20 |
| | | | | 14759/60 | a | 35 | 25 |
| | | | | Average | | 50.0 | 28.1 |

in the case of the fresh tissue extracts by a layer of fat which often deposited on the cellophane tubing.

(c) Reconstitution of the cell growth promoting activity of the dialyzed extract from acetone-desiccated chicken hearts.

Under the conditions of the experiment, the dialysate could be con-

sidered to have contained practically the same concentration of dialyzable substances as the original heart extract, since as little as 20 mls. of Tyrode's solution were dialyzed against 12 times as much heart extract; similarly the dialyzed extract necessarily contained the same concentration of non-dialyzable substances as the original extract, as there was no change in the volume of the dialyzed extract during dialysis. Therefore, if there were a complete reconstitution effect, one should obtain, by mixing one volume of dialyzed extract with one volume of the corresponding dialysate, a solution showing the same growth promoting effect as a 50% dilution with Tyrode's solution of the original undialyzed heart extract.

The reconstitution experiments performed with 3 and 7 days dialyzed heart extract and the corresponding dialysates are summarized in Tables 7 and 8. In every case there was a complete reconstitution of the original effect.

TABLE 7

I. THE AREA IN MM² OF 6 DAYS OLD FIBROBLAST COLONIES (SISTER HALVES) GROWING IN THE PRESENCE OF 50% EXTRACT FROM ACETONE DESICCATED HEARTS (A), AND OF A MIXTURE OF EQUAL VOLUMES OF EXTRACT FROM ACETONE DESICCATED HEARTS, DIALYZED FOR 3 DAYS, WITH THE CORRESPONDING DIALYSATE (B).

| No. of cultures | | A | B | No. of cultures | | A | B |
|---|---|---|---|---|---|---|---|
| 14731/32 | a | 54 | 40 | 15139/40 | a | 63 | 60 |
| | b | 80 | 80 | 15141/42 | a | 39 | 45 |
| 14735/36 | a | 61 | 40 | | b | 50 | 42 |
| | b | 45 | 43 | 15143/44 | a | 45 | 50 |
| 14737/38 | a | 68 | 75 | 15145/46 | a | 64 | 64 |
| | b | 80 | 83 | | | | |
| 15137/38 | a | 70 | 60 | Average | | 59.9 | 56.8 |

TABLE 8

I. THE AREA IN MM² OF 6 DAYS OLD FIBROBLAST COLONIES (SISTER HALVES) GROWING IN THE PRESENCE OF 50% EXTRACT FROM ACETONE DESICCATED HEARTS (A), AND OF A MIXTURE OF EQUAL VOLUMES OF EXTRACT FROM ACETONE DESICCATED HEARTS, DIALYZED FOR 7 DAYS, WITH THE CORRESPONDING DIALYSATE (B).

| No. of cultures | | A | B | No. of cultures | | A | B |
|---|---|---|---|---|---|---|---|
| 14843/44 | a | 112 | 102 | 15151/52 | a | 45 | 50 |
| 14847/48 | a | 100 | 109 | 15155/56 | a | 50 | 54 |
| | b | 111 | 109 | | b | 53 | 57 |
| 14927/28 | a | 55 | 61 | 15159/60 | a | 66 | 59 |
| 14929/30 | a | 65 | 64 | 15161/62 | a | 61 | 57 |
| 15147/48 | a | 50 | 56 | | | | |
| | b | 48 | 50 | | | | |
| 15149/50 | a | 88 | 87 | | | | |
| | b | 46 | 45 | Average | | 67.8 | 68.6 |

## Discussion

A number of workers investigating the chemical nature of cell growth promoting embryonic extracts have studied the dialyzability of the active factors present in this material.*

Wright (1925) stated that the principle in the embryonic tissue extracts which "excites mitosis" passes freely through a collodion membrane. Using fresh embryonic chick heart explants, he estimated the growth promoting activity of the extracts by counting the total number of mitoses in the outgrowth area.

Baker and Carrel (1926) found that dialysis greatly reduced but did not abolish entirely the growth activating effect of embryonic extract. The rate of growth of fibroblast colonies carried through several passages was consistently greater in the dialyzed extract than in Tyrode's solution.

Iwasaki (1936) found that the growth of iris epithelium in vitro could not be kept up for a long time in a medium consisting of dialyzed plasma and dialyzed embryonic extract..

Jacoby (1937) found that dialysis of embryonic extract reduces its effect on the migratory activity of fibroblasts, as measured by the radial extension of the cultures, and on the mitotic activity of the cells. The dialysate had an effect slightly greater than a physiological salt solution, but far smaller than the dialyzed extract.

Tazima (1940) found that dialysis of chick embryonic extract caused it to lose some of its growth activating power, which, however, could be reconstituted by adding the dialysate to the dialyzed extract.

In the experiments presented in this paper it was shown:

a) that the cell growth promoting activity of adult chicken heart extract (both from fresh tissue and from acetone-desiccated material) can be greatly reduced by dialysis,

b) that the dialysate contains very little activity of its own (extracts of acetone-desiccated material) or none at all (extracts of fresh tissue),

c) that combining the dialyzed extract of acetone-desiccated material with the dialysate brings about a complete recovery of the activity of the dialyzed extract.

*It may be noted that the findings of Kimura (1938), Tazima (1940) and Fischer (1941) who dialyzed plasma, the other basic constituent of the tissue culture medium, parallel those reported for embryonic extracts.

The results we have obtained with the dialysis of a cell growth promoting adult tissue extract are in agreement with those previously reported by the majority of authors who have applied this procedure to embryonic extracts. This suggests that the cell growth promoting principles present in adult and embryonic tissue extracts are of a similar nature.

From the experiments described in the present paper, it seems justifiable to conclude that the cell growth promoting activity of adult tissue extracts depends on at least two factors, or two series of factors, each of which requires the presence of the other in order to manifest its full effect.

## Summary

The effect of dialysis on the "growth-promoting" activity of adult tissue extracts was determined on fresh adult chicken heart extract and on extract of acetone-desiccated adult chicken hearts.

1. In the case of fresh heart extract it was found that:
   a. dialysis greatly reduces the growth-promoting activity;
   b. the dialysate has no activity.
2. In the case of extract of acetone-desiccated hearts, it was found that:
   a. dialysis reduces the growth-promoting activity to less than 25% of its original value;
   b. the dialysate has only slight activity when tested alone;
   c. combining the dialyzed extract with the dialysate causes a complete recovery of the original activity.

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*Growth*, 1948, **12**, 15-32

# THE GIGANTISM PRODUCED IN NORMAL RATS BY INJECTION OF THE PITUITARY GROWTH HORMONE

## *I.—Body Growth and Organ Changes*[1]

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Gigantism was first induced experimentally in the rat by the injection of anterior pituitary extracts by Evans and Long in 1921. A growth promoting hormone in the extract was postulated as the cause of the excess growth. During the years in which in this laboratory the growth hormone was gradually freed of other contaminating active principles found in the anterior lobe, this phenomenon of an overgrowth of the normal body was steadily confirmed. However, doubt arose as to the existence of a separate pituitary principle responsible for growth, partly due to the fact that in some animals other pituitary principles appeared to affect growth. Furthermore, it was found that the growth was sometimes not continuous on chronic administration of pituitary extracts, and the possibility of the development of a specific anti-growth factor was discussed. With the isolation of the pure growth hormone (Li, Evans and Simpson, 1945), further repetition of the chronic administration of the principle became necessary, in order to establish the biological properties of this hormone when freed from other anterior hypophyseal substances. Specifically it was sought to ascertain: the hormone's effect on body growth, both in weight and dimensions; its possible differential effect on organ weights; its effect on the microscopic structure of the organs, especially the endocrine organs; its effect on osteogenesis; and some of its effects on the chemical composition of the body. These topics will be presented in a series of separate papers.[2]

[1]Aided by grants from the American Cancer Society, Inc., Research Fund, New York City, the United States Public Health Service (RG-409), Washington, D. C., and the Board of Research of the University of California, and the Rockefeller Foundation, New York City.

[2]The second paper of this series deals with the histology of the pituitary, the third

In this paper, dealing with general somatic growth, the main objectives were as follows: (1) To determine whether body growth (weight and length increase) could be maintained for long periods; (2) to determine, provided growth continued, whether it would be maintained at the initial rate or gradually diminish; (3) to determine whether the growth of organs would be proportional to body growth or would conform to the pattern seen in normal development, namely that organs serving the whole body such as the kidney would increase in proportion to the body, whereas other organs such as the reproductive and endocrine organs would follow a distinctive growth pattern; (4) to see whether any of the organs, especially endocrine and reproductive organs, showed any morphological changes indicative of hormonal contamination of the growth promoting preparation.

Female rats which had previously attained growth stasis (192-221 days old) were selected for the experiment. They were maintained on a complete diet (diet XIV)[3] supplemented by lettuce. The growth hormone preparation used, L1389P, contained 50 units per mg, as assayed in hypophysectomized immature female rats (0.02 mg daily caused an average body weight increase of 10 gm in 10 days). Injections of growth hormone were given to 15 rats; another 15 were retained as uninjected controls. Of these, 8 injected and 4 controls were maintained for an injection period of 437 days. The injections were given daily 6 days of the week. The initial dose of growth hormone was 0.4 mg. This dose was increased several times during the injection period so that the dose would remain approximately proportional to body weight (0.4 mg for 24 days, 0.6 mg for 68 days, 1.0 mg for 34 days, 1.5 mg for 115 days, 2.0 mg for 196 days). The injections were given intraperitoneally in 0.5 cc of 0.85% saline. The animals were weighed at 5 day intervals; tails (anus to tail tip) were measured at intervals to give an indication of the rate of skeletal increase.

The size ultimately attained by the injected rats as compared with their controls is shown in Figures 1 and 2. Body weight increases of the injected and control groups during the period of the experiment

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with some changes in chemical composition of the body, the fourth with skeletal changes in the tibia, costochondral junction and caudal vertebrae, the fifth with changes in the skull and dentition.

[3]Composition of diet XIV: Whole wheat 68.5%, casein 5%, fish meal 10%, alfalfa leaf meal 10%, NaCl 1.5%, fish oil 5%.

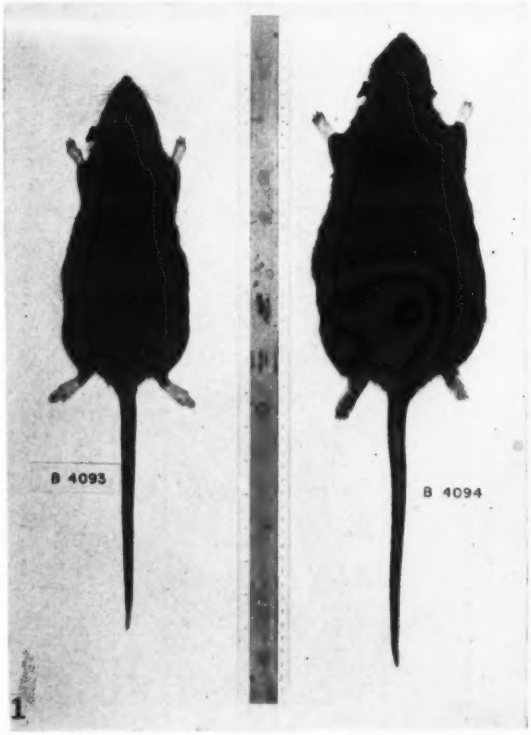


FIGURE 1

(Right) GROWTH HORMONE INJECTED RAT, BODY WEIGHT 662 GM, TOTAL LENGTH 48.2 CM, AGE 630 DAYS
(Largest of rats receiving injections)
(Left) UNINJECTED LITTERMATE CONTROL, BODY WEIGHT 322 GM, TOTAL LENGTH 42.9 CM
Photographed under ether anesthesia at the termination of the experiment.

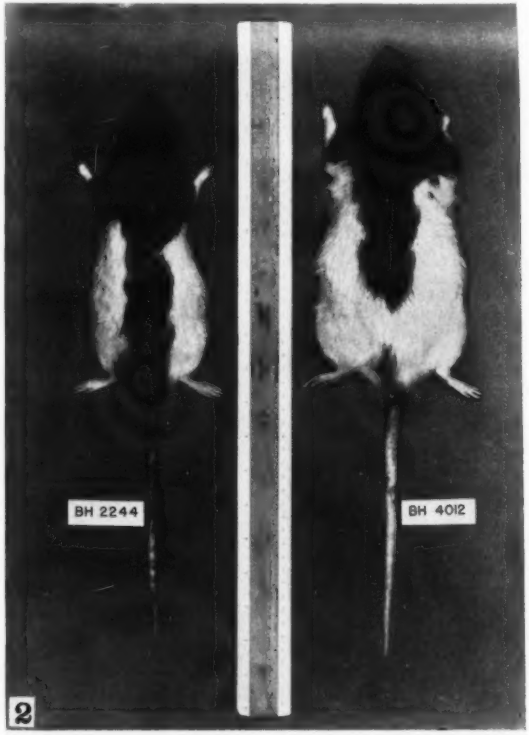


FIGURE 2

(Right) GROWTH HORMONE INJECTED RAT, BODY WEIGHT 428 GM, TOTAL LENGTH 43.0 CM, AGE 630 DAYS
(Smallest of rats receiving injections)
(Left) UNINJECTED CONTROL BODY WEIGHT 281 GM, TOTAL LENGTH 41.0 CM, AGE 658 DAYS
Photographed under the same conditions as the rats in Figure 1.

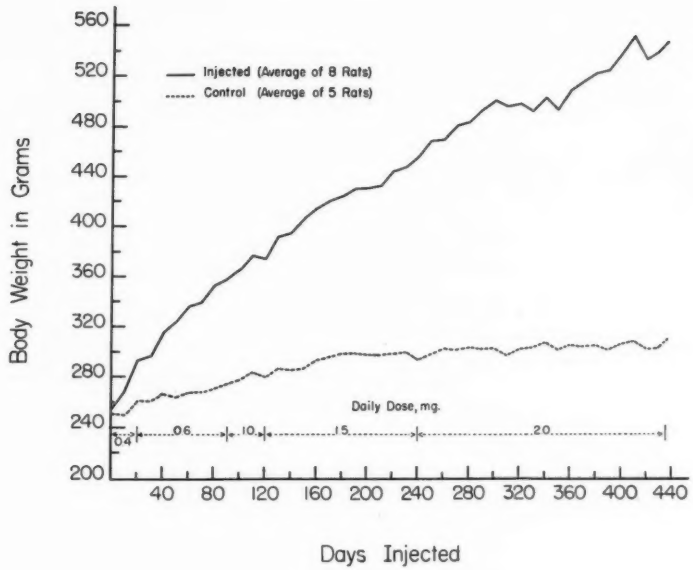


FIGURE 3

CURVES SHOWING THE AVERAGE GROWTH OF 8 GROWTH HORMONE INJECTED AND 5 UNINJECTED RATS DURING THE 437-DAY EXPERIMENTAL PERIOD.

TABLE 1

BODY WEIGHT AND LENGTH OF FEMALE RATS INJECTED FOR 437 DAYS WITH PITUITARY GROWTH HORMONE

(From 210 to 647 days of age)

| Treatment | No. of rats | Body Weight | | | Length at Autopsy[2] | | |
|---|---|---|---|---|---|---|---|
| | | Onset | Autopsy | Gain | Nose to anus | Anus to tail tip | Total |
| | | gm | gm | gm | cm | cm | cm |
| Growth Hormone | 8 | 255 ± 6.09[1] | 548 ± 30.59 | 293 ± 27.27 | 24.3 ± 0.28 | 21.1 ± 0.38 | 45.4 ± 0.60 |
| Control uninjected | 4 | 229 ± 11.78 | 288 ± 11.47 | 59 ± 11.92 | 21.9 ± 0.42 | 18.7 ± 0.52 | 40.5 ± 0.93 |

[1]The figures preceded by ± are the Standard Errors. A difference between means 3 times the standard deviation of the difference between those means is considered highly significant, 2 times is considered barely significant, less is not significant.

[2]Measurements made on rats under amytal anesthesia.

are shown in Fig. 3. The average weight of the experimental and control groups at the beginning and end of the experiment can be seen in Table 1; also total body lengths of the two groups of rats at autopsy can be compared. Although considerable variation in body weight increases occurred, there was no overlapping of the rate of gain of controls and injected rats, as can be seen in Figure 4, where

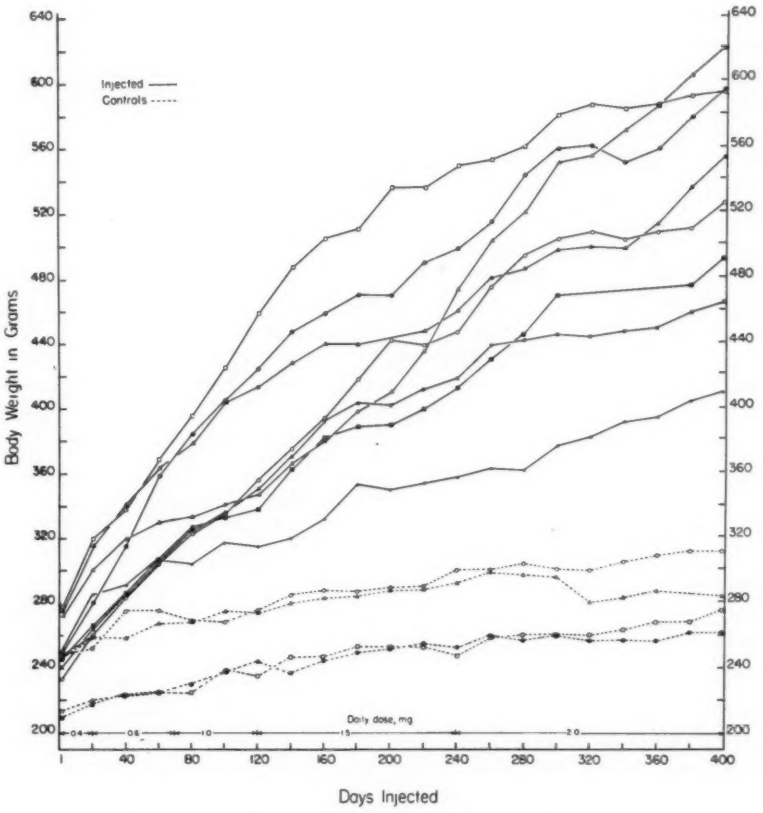


FIGURE 4

CURVES SHOWING THE GROWTH OF INDIVIDUAL ANIMALS (8 INJECTED GROWTH HORMONE AND 4 UNINJECTED RATS) DURING 400 DAYS OF THE EXPERIMENTAL PERIOD. Note that there is no overlapping between the curves of injected and uninjected rats.

individual rates of gain are plotted. The body weight of the controls increased only slightly, the average gain during the 437 day injection period being 59 gm. The least responsive of the growth hormone injected animals gained three times this amount (177 gm) and the most responsive gained almost seven times as much (380 gm). There was also no overlap in the increase in tail lengths of experimental and control rats nor in the total body lengths at autopsy. The proportion of body length to tail length remained the same in the experimental as in the control group.

It is quite clear from the curves (Figures 3 and 4) that acceleration of growth in response to injections began at once and continued throughout the prolonged experimental period. The animals were still growing at the end of the experimental period, even though by this time they were of advanced age. The rate of growth in terms of weight gain per unit of time, however, gradually decreased. The curves based on averages (Figure 3) show that the initial growth rate, in terms of grams gain per 10 days, was 22 gm; this rate was halved by 20 days (11 gm per 10 days); by 70-80 days it was a fifth this rate (4 gm per 10 days). Part of the decline in rate of growth may have been due to inadequate adjustment of the dose to the increasing body weight. It was noted that some enhancement of growth rate did occur when the dose levels were raised. The rate of growth of the experimental rats was greater at all times than that of the controls.

The skin of the injected rats grew in excess even of the amount required to cover their greater bulk, as could be demonstrated by the greater folds of pelt which could be plucked up from injected than from control rats.

## Viscera

Table 2 shows the weights of those viscera which, judging from their response during normal growth epochs, might be expected to increase in weight in proportion to body weight. It will be seen that the weights of the liver, kidneys, heart, stomach and intestine of injected rats did keep pace with the increased body weight. The absolute weights of these organs were greatly increased but the weight in grams per 100 gram body weight was practically the same as in the controls.

TABLE 2
WEIGHTS OF VISCERA

| Treatment | No. of rats | Body weight at autopsy | Organ Weights | | | | |
|---|---|---|---|---|---|---|---|
| | | | Liver | Kidneys | Heart | Stomach | Intestine |
| | | gm | gm | gm | gm | gm | gm |
| Growth Hormone | 8 | 548 ± 30.59 | 24.31 ± 1.83 | 3.35 ± 0.19 | 1.46 ± 0.12 | 2.47[1] ± 0.09 | 14.24 ± 0.91 |
| | | gm per 100 gm body weight: | 4.44 | 0.61 | 0.26 | 0.45 | 2.60 |
| Control uninjected | 4 | 288 ± 11.47 | 13.36 ± 0.43 | 2.03 ± 0.08 | 0.94 ± 0.07 | 1.46 ± 0.06 | 8.37 ± 0.21 |
| | | gm per 100 gm body weight: | 4.67 | 0.71 | 0.33 | 0.57 | 2.91 |

[1]Average of 6 injected rats.

The increased size of the kidney could be seen from microscopic examination to be due, at least in part, to the increased diameter of the tubules and the glomeruli.[4] The liver cells were large but the size of the cells did not offer an adequate explanation of the greatly increased liver size.

The pancreas, though not weighed, due to its diffuse distribution, could be seen to be increased in size. A piece of pancreatic tissue was fixed in Zenker-formol at once on opening the peritoneal cavity and sectioned at 4 micra after imbedding in nitro-cellulose. The sections were stained by the modified Mallory-azan technique used for study of the pituitary.[5] It was noted that the islets were increased in size in the injected rats. The beta cells in them were noticeably larger. The negative image of the Golgi apparatus could be seen clearly in almost all the beta cells of the experimental animals and appeared to be somewhat larger than in these cells of the controls. No perceptible changes were noted in the granular content of the cells. The alpha cells did not present any variation from normal either in number, general appearance or distribution.

[4]Average diameter of Bowman's capsule, based on measurement with an ocular micrometer of ten glomeruli per rat, was 498 μ in 8 injected and 412 μ in 4 control rats. The average diameter of the proximal convoluted tubules, based on the same number of measurements per rat, was 157 μ in the experimental group, 124 μ in the control group.

[5]The technical procedure and analysis of the pancreas was conducted by Dr. A. A. Koneff.

## Endocrine and Reproductive Organs

The absolute weights of the ovaries, uteri, preputials, adrenals and pituitaries were only slightly increased in the injected rats (Table 3). These increases were statistically significant only in the case of the preputials, adrenals, and pituitaries. The average thyroid weight was less in the injected than in the control group, but the difference was not significant. The thymi were small, but not as completely atrophic in most of the injected rats as in the controls. (The thymi could be recognized and dissected in 6 injected and in only 2 control rats.) All these endocrine and reproductive organs constituted a smaller per cent of the body weight in the experimental than in the control animals.

TABLE 3
Weights of Reproductive and Endocrine Organs

| Treatment | No. of rats | Body weight at autopsy | Weights of Reproductive Organs | | | Weights of Endocrine Organs | | |
|---|---|---|---|---|---|---|---|---|
| | | | Ovary[1] | Uterus[2] | Preputials | Thyroid | Adrenals | Pituitary[4] |
| | | gm | mg | mg | mg | mg | mg | mg |
| Growth Hormone | 8 | 548 ± 30.59 | 36 ± 4.90 | 1079 ±141.33 | 103 ± 13.75 | 37 ± 2.97 | 99[3] ± 6.72 | 21.1 ± 2.10 |
| | | mg per 100 gm body weight: | 6.6 | 197 | 19 | 6.7 | 18 | 3.8 |
| Control uninjected | 4 | 288 ± 11.47 | 24 ± 2.57 | 871 ±209.11 | 66 ± 6.67 | 42 ± 7.55 | 62 ± 3.23 | 14.1 ± 0.85 |
| | | mg per 100 gm body weight: | 8.3 | 321 | 23 | 14.6 | 21 | 4.9 |

[1]Weight of one ovary, other fixed directly within bursa. Average of 7 injected rats.
[2]Includes weight of one oviduct, other fixed with ovary.
[3]Average based on 6 rats; one case with medullary tumor, another with hemorraghic cortex, not included.
[4]Whole pituitary weighed after Zenker-formol fixation.

The endocrine and reproductive organs were examined microscopically for evidence of change. Hematoxylin and eosin, Mallory-azan, iron hemotoxylin aniline blue, and Sudan orange stains were used as indicated by the tissue and findings.

### *Thyroids and Parathyroids*

The morphology of the thyroid of injected rats was not distinguishable from that of controls (Figures 5 and 6). The height of the epithelium and the size of the follicles were about the same. Meta-

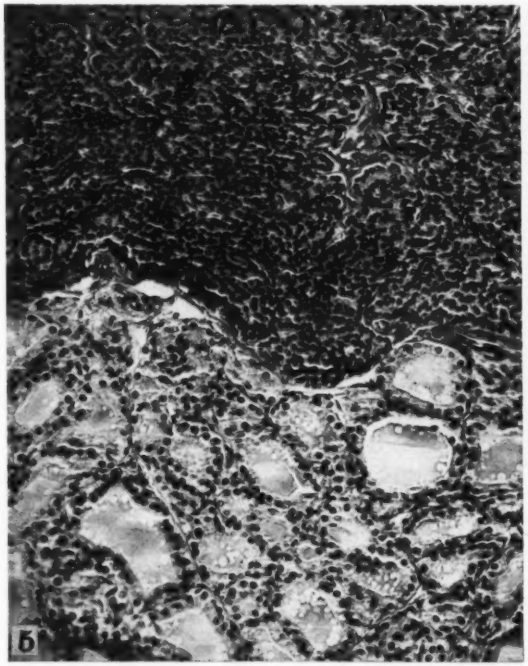


FIGURE 5
THYROID AND PARATHYROID OF NORMAL 630 DAY OLD RAT. WEIGHT OF THYROID 63 MG. H. AND E. X 180.

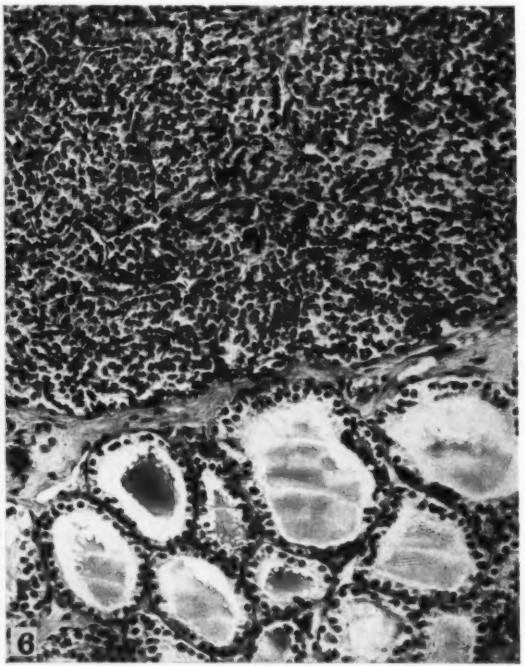


FIGURE 6
THYROID AND PARATHYROID OF 630 DAY OLD RAT INJECTED WITH GROWTH HORMONE FOR 437 DAYS. WEIGHT OF THYROID 41 MG. H. AND E. X 180.
(These thyroids are from the animals shown in Figure 1)

plastic areas were observed in the epithelium, but these areas of changed epithelium occurred in about the same proportion of controls and experimental rats (3 of 8 growth hormone-treated, 2 or 4 controls). These metaplastic changes were therefore probably related to the age of the animals. The parathyroid cells seemed clearly larger and their pseudo-alveolar arrangement more evident in some members of the injected group.

### *Adrenals*

The adrenals were studied with hematoxylin and eosin, and Sudan orange stains. The adrenal cortices of the injected rats did not appear to be markedly affected by the treatment. They showed the normal, uniform distribution of lipid except for a tendency toward depletion in the outer and inner cell layers of the glomerulosa. This was in no way comparable to formation of a subglomerular sudanophobe zone. Nodular cortical masses which stained heavily with lipid stains occurred in a somewhat greater number of injected than in uninjected rats. The medullas of the adrenals were slightly larger in proportion to total adrenal size in the injected rats than in the controls.

### *Reproductive System*

The reproductive systems in the controls were quite normal in spite of the advanced age. Most of them were still having normal vaginal cycles.[6] In all but 2 controls the ovaries (Figure 7) contained several sets of corpora lutea, indicating that ovulation was occurring rhythmically. Follicles only (some being cystics) were found in the ovaries of the two non-ovulating animals. The reproductive systems of the rats receiving injections were considerably disturbed. Two only were having fairly regular vaginal cycles, and in these, new and old corpora lutea were present in the ovaries. Corpora lutea were also present in the ovary of a third animal in which the cyclic vaginal changes had been obscured by the presence of infection. The ovaries of the other 5 injected rats contained only a few small follicles, or follicular cysts. An outstanding feature of these ovaries was the presence of structures resembling atrophic testicular tubules. These were apparently derived from transforma-

[6]At least two showed evidence of infection in vagina or uterus. One animal (which had to be sacrificed 3 weeks before the rest of the group) showed profuse bleeding from the vagina, which was traced to a cystic glandular hyperplasia of the uterus.

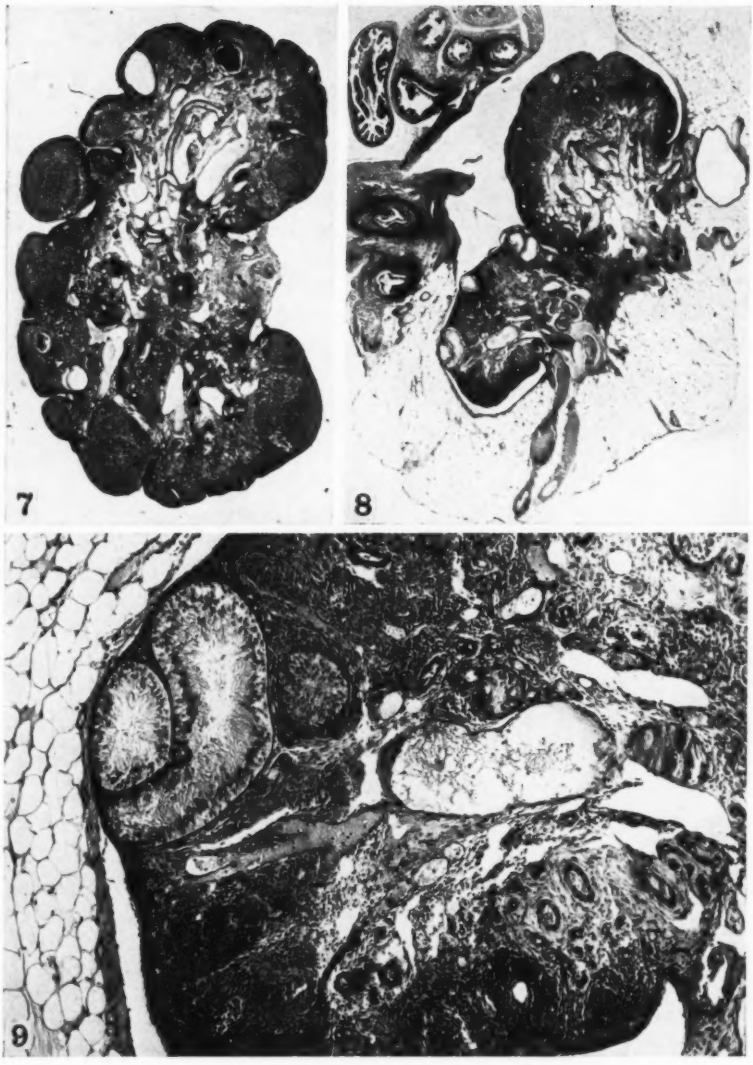


FIGURE 7

OVARY OF NORMAL 630 DAY OLD RAT

Cyclic changes were occurring, as indicated by the vaginal smear during life and by the presence of fresh corpora lutea in the ovary at the time of autopsy. Weight of contralateral ovary 27 mg. H. and E. X 13.

FIGURE 8

OVARY OF 630 DAY OLD RAT INJECTED FOR 437 DAYS WITH GROWTH HORMONE

Note absence of follicles and corpora lutea, also the tubular transformation in the cortical region. Weight of contralateral ovary 52 mg. H. and E. X 13.
(This is the injected animal illustrated in Figure 1)

FIGURE 9

HIGHER MAGNIFICATION (X 83) OF PART OF THE SECTION SHOWN IN FIGURE 8

Note filamentous cytoplasm of the cells lining the cortical tubular structures.

tions of small follicles. They were not confined to the hilum and therefore cannot properly be called hilum tubules. Typical hilum tubules were also present in abundance. (Areas showing tubular transformation also occurred in cyclic ovaries of the group.) Figure 8 shows a section of one of the ovaries of a non-ovulating animal in which tubules resembling atrophic testicular tubules can be seen in the cortical area. Figure 9 shows a higher magnification of these tubular structures. Figure 10 shows a group of such "testicular tubules" in an ovary containing a follicular cyst. Also present in this section near the oviduct was an epididymis like structure. Figure 11 shows a higher magnification of the group of tubules near the loop of oviduct and Figure 12 shows a higher magnification of the tubular structures in the ovary.

Interstitial cells were difficult to recognize in some of these ovaries of injected rats, being replaced over large areas by peculiar light staining cells. Figure 13 shows such a homogeneous light staining area. There is a suggestion here of the formation of numerous minute tubular structures. Only a few small active follicles were present in this atrophic ovary. A similar transformation of the ovary has been reported and illustrated by Evans, Simpson and Pencharz (1939) as present in old thyroidectomized rats.

The germinal epithelium was fat laden in some ovaries of the injected rats. Extensive indipping and pinching off of germinal epithelium was observed in many places. The ovary illustrated in Figure 13 exhibited this behavior of the germinal epithelium—here almost resembling villi. A higher magnification of this area is shown in Figure 14.

The mammary glands[7] of the normal animals consisted of ducts and a few alveolar buds (DAB) as shown in Figure 15. A few scattered distended alveoli were observed in the glands of 3 rats. In the injected rats, on the other hand, lobulo-alveolar development occurred in almost all rats, but the degree of development varied greatly: in terms of the abbreviations here employed, LA 1+ to 2+ occurred in two rats with scattered instances of alveolar distension, LA 2+ to 3+ in two rats, LA 4+ in two rats. The other two injected rats showed duct and alveolar bud development (DAB) which was within control

[7]Mammary spreads were made by stripping the glandular tissue from the skin and musculature (usually in the right inguinal region). They were fixed in formol, stained with alum-carmine, cleared in methyl salicylate, and photographed by transmitted light.

FIGURE 10

OVARY OF 630 DAY OLD RAT INJECTED FOR 437 DAYS WITH GROWTH HORMONE
In addition to a cyst, this ovary contains a mass of tubular structures resembling testicular tubules. Near the oviduct is a mass of tubules resembling an epididymis. Weight of contralateral ovary 49 mg. H. and E. X 13.

FIGURE 11

HIGHER MAGNIFICATION (X 143) OF TUBULES NEAR OVIDUCT, SHOWN IN FIGURE 10

FIGURE 12

HIGHER MAGNIFICATION (X 143) OF TESTIS-LIKE TUBULES WITHIN THE OVARY SHOWN IN FIGURE 10

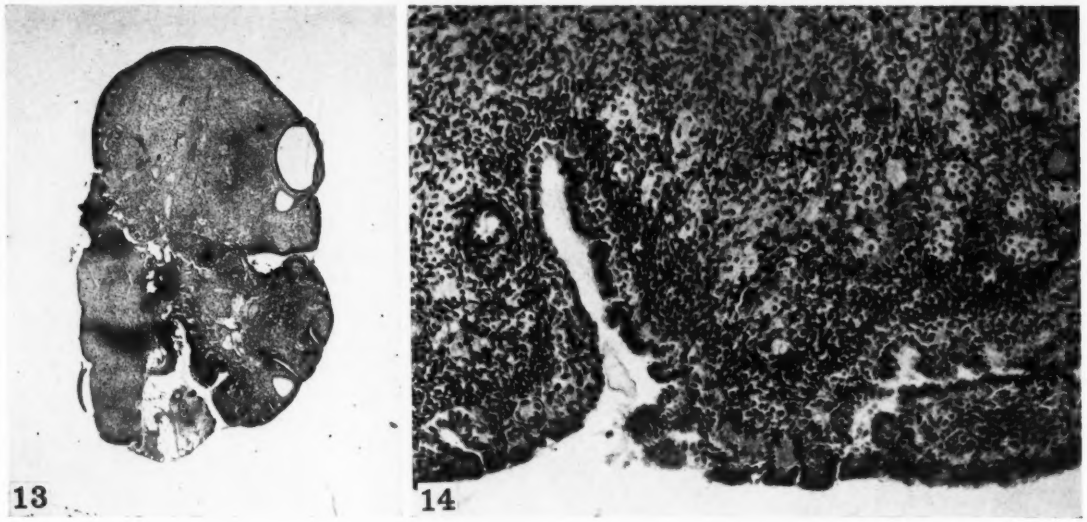


FIGURE 13
OVARY OF GROWTH HORMONE INJECTED 658 DAY OLD RAT SHOWING HOMOGENEOUS CHARACTER OF MOST OF THE ORGAN
Weight of contralateral ovary 25 mg. H. and E. X 13.

FIGURE 14
HIGHER MAGNIFICATION (X 143) OF THE GERMINAL EPITHELIUM OF THE OVARY IN FIGURE 13, SHOWING MARKED INDIPPINGS

FIGURE 15
Mammary gland of normal 630 day old rat, showing only ducts with a few alveolar buds (DAB). (The ovary of this rat is shown in Figure 7.) X 2.5.

FIGURE 16
Mammary gland of 658 day old rat injected for 437 days with growth hormone. Lobulo-alveolar development (LA +2) is present. There is also a marked widening of the ducts. X 2.5.

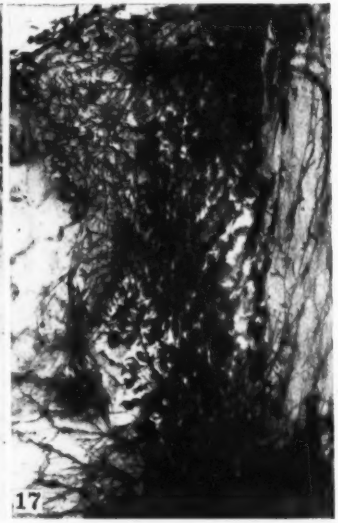


FIGURE 17
Mammary gland of 630 day old rat injected for 437 days with growth hormone. Lobulo-alveolar development of +4 contrasts with undeveloped adjacent areas. X 2.5. (This is the injected animal illustrated in Figure 1. The atrophic ovaries are shown in Figures 8 and 9, and the thyroid in Figure 6.)

limits. The wide ducts present in one animal with LA 1 to 2+ should be noted and are illustrated in Figure 16. In the glands described as characterized by lobulo-alveolar development of 2+ to 3+, the development attained was frequently quite uniform throughout the gland. In the animals where LA 4+ was attained the reaction characterized only part of the gland (Figure 16).

### Tumor Formation and Other Abnormalities

Tumors of mammary origin (fibromata or adenofibromata) were observed in three of the experimental rats; none occurred in the uninjected rats. It is to be noted that marked mammary development was observed (LA 2+ to 3+ and 4+) in rats where cyclic phenomena were not occurring, as judged by absence of large follicles and corpora lutea, and that mammary tumor formation was also observed in one rat whose ovaries were atrophic. In the other two rats in which mammary tumors were present, the ovaries contained corpora lutea. The tumors were multiple in some animals, occurring both in the inguinal and axillary region. All were lobular tumors. In some lobules acinar tissue had completely disappeared. The fibrous tissue was usually dense and in distribution reflected the pattern of the original organ even when acinar tissue had disappeared; in some the stroma was less fibrous and growing very actively judged by the irregularity in size of cells, their youthful appearance and the frequency of mitoses. All the tumors were encapsulated.

Other types of tumors beside mammary tumors were also observed in the injected rats. Multiple fibrous tumors of the skin were present in dorsal areas of at least one rat. Their position obviated their mammary origin or their origin from injection sites. In another injected rat a large fibrous tumor occurred in the sub-diaphagmatic region of the abdominal cavity. This tumor resembled the more fibrous parts of the mammary tumors and it, too, suggested malignancy. This was the only tumor which penetrated its capsule.

Tumors of the ovary were present in three injected rats; none were observed in controls though serial sections were made of one complete ovary of each animal. The tumors were small, even the largest being not much larger than the corpus luteum of pregnancy. It was difficult to determine whether these tumors were derived from theca or granulosa cells (similarities to both having been observed in the different

tumors) or whether they could more properly be compared with the less differentiated type of human arrhenoblastoma cell.

A large tumor was present in the center of the adrenal of one injected rat. The adrenal containing the tumor weighed 549 mg. The contralateral adrenal weighed 54 mg. The cortex of the tumor-containing adrenal was stretched in a thin layer over the tumor. The epithelial cells of which the tumor was composed resembled medullary cells except that they were larger. The tumor had compressed the remains of the medulla to one side and the tumor cells were continuous with the medullary cells.

The tumors described occurred only in the injected rats. It should be noted, however, that mammary tumors have been observed in old normal females of the strain used (Long-Evans). None happened to have occurred in the controls of this series. None of the other types of tumors noted above occurred in the controls. However, two uninjected rats, which had been retained as controls but which died shortly before the termination of the experiment, had tumors: One had a tumor between the rectum and bladder, suggestive of mesothelioma;[8] another had a thickened intestinal wall due apparently to tumorous growth in the longitudinal smooth muscle coat. Another control was eliminated from the series because of a pituitary adenoma.

Although it appears necessary to report an increased incidence of tumor formation in these experimental animals injected with growth hormone, hasty conclusions would be regrettable. A similar experimental series conducted on hypophysectomized rats for the same length of time showed no tumor formation resulting from injection of growth hormone, and it might seem justified to attribute tumor formation to an indirect effect of the growth hormone acting through the animals' pituitary. However, these results are in urgent need of verification with even higher dose levels of the hormone and prolonged experimentation.

## SUMMARY

1. Chronic injection of growth hormone into normal plateaued female rats resulted in continuous growth for a 437 day period, or

[8]Thanks should be extended to Dr. Henry D. Moon, pathologist at Letterman Hospital and University of California Hospital, San Francisco, who examined all tumors observed in this series.

until the animals were 647 days of age. The rate of growth was not, however, as rapid at the end of the period as at the beginning.

2. The weights of viscera (liver, stomach, intestine, kidney and heart) increased markedly and maintained the same proportion to the body weight as in the controls.

3. The reproductive organs decreased in weight and thus constituted a much smaller percentage of the body weight than in controls.

4. The endocrine organs all weighed less in proportion to body weight than in the normal controls. The adrenals and pituitaries were slightly increased in absolute weight, the thyroids slightly decreased.

5. Histologically the thyroids, adrenals and ovaries showed no evidence of specific hormonal stimulation. The ovaries were seriously hurt in the injected rats.

6. The mammary glands showed an unexplained lobulo-alveolar development.

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*Growth*, 1948, **12**, 33-37

# THE GIGANTISM PRODUCED IN NORMAL RATS BY INJECTION OF THE PITUITARY GROWTH HORMONE

## *II. Histological Changes in the Pituitary*[1]

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(Received for publication January 6, 1948)

The preceding paper in this series described the production of gigantism by chronic administration of the pituitary growth hormone to normal, plateaued female rats (Evans, Simpson and Li, 1948). This paper is devoted to the histology of the pituitaries of these giant rats. No study has been found of the effect of the growth hormone on the pituitary of the rat, but Franck (1937) studied the effect in the guinea pig of administration of a growth-promoting preparation.[2] He has stated that typical acidophils completely disappeared, and that small acidophils and chromophobes were increased in number. He also reported a decrease in basophils.

The detailed account of the conditions of this experiment, in which pure growth hormone has been injected into rats, has already been given. In brief, it may be said that the average age at the beginning of the injections was 210 days, at autopsy 647 days. The growth hormone was administered intraperitoneally, six days weekly, in doses gradually increasing from 0.4 mg to 2.0 mg. Pituitaries from eight growth hormone injected rats and four controls were examined. The pituitaries were fixed in Zenker-formal and sectioned at 4 micra after nitro-cellulose embedding. Sections were stained by a modified Mallory-azan technique (Koneff, 1938).

The average weight of the pituitaries in the experimental group

[1]Aided by grants from the American Cancer Society, Inc., Research Fund, New York City, the United States Public Health Service (RG-409), Washington, D. C., and the Board of Research of the University of California, and the Rockefeller Foundation, New York City.

[2]The growth stimulating principle was prepared by the Leo Chemical Works, Copenhagen.

was greater than that in the control group (21 mg in experimental, 14.0 mg in control rats). They did not, of course, increase in proportion to the body weight.

*Acidophils*

Although no differential counts were made, the number of acidophils in the pituitaries of the injected rats appeared to be considerably decreased. Planimeter measurements made of camera lucid tracings of acidophils showed that their size was also decreased. As shown in Table 1, the average size of the acidophils was 156 planimeter units,

TABLE 1
RELATIONSHIP BETWEEN SIZE OF ACIDOPHILS AND BODY WEIGHT GAIN IN RATS INJECTED CHRONICALLY WITH GROWTH HORMONE

| Group | Size of Acidophils | Weight Gain |
|---|---|---|
| | Planimeter units* | grams |
| Injected | 144 | 390 |
| | 135 | 356 |
| | 145 | 346 |
| | 149 | 327 |
| | 173 | 295 |
| | 153 | 257 |
| | 184 | 195 |
| | 164 | 177 |
| Ave. | 156 ± 6** | 293 ± 27 |
| Control | 198 | 75 |
| | 170 | 71 |
| | 187 | 62 |
| | 200 | 29 |
| Ave. | 188 ± 7 | 59 ± 11 |

*An arbitrary unit. One hundred cells were outlined with a camera lucida and the area measured with a planimeter.
**The figures preceded by ± are the Standard Errors.

whereas in the controls it was 188 units. This difference was statistically significant. The size of the acidophils was most markedly decreased in the pituitaries of those rats which gained the most in body weight.

A decreased granular content also characterized the acidophils of the injected rats. In many instances, the nucleus was surrounded merely by a rim of cytoplasm containing a single row of granules. The impression was gained that no new granular material was being formed in the cytoplasm, and that the cells were being depleted of

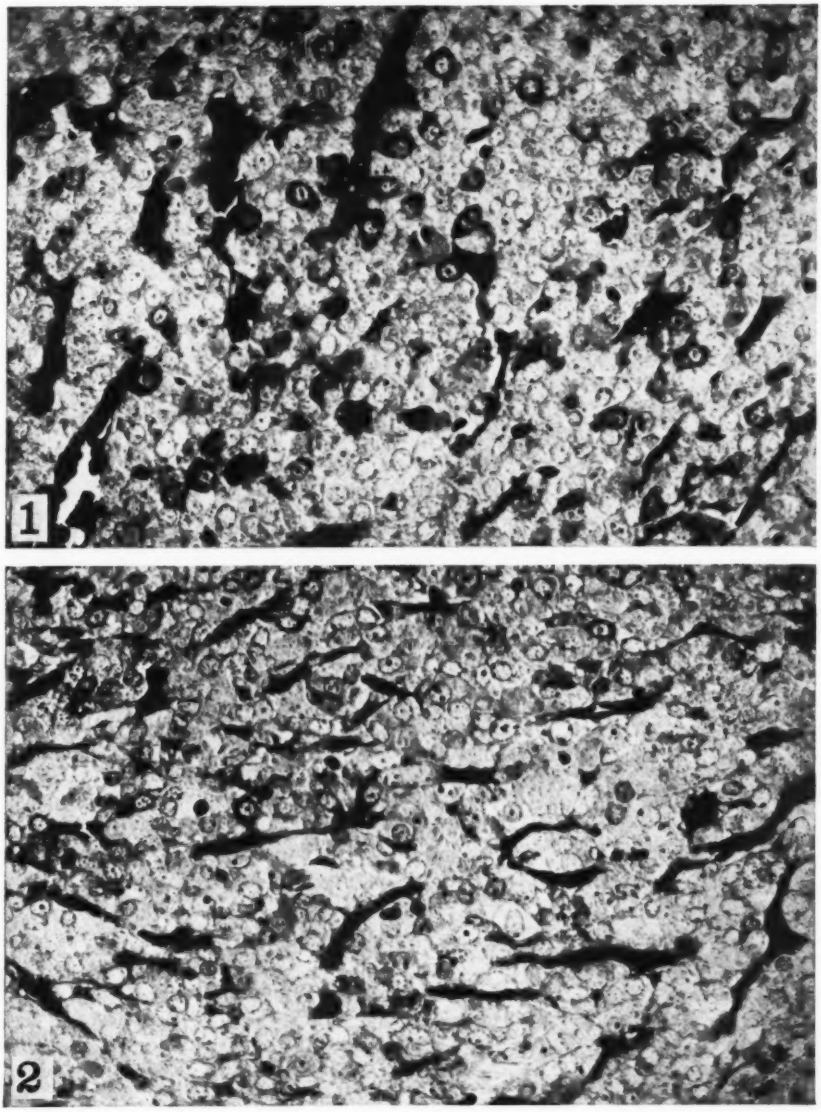


FIGURE 1

PHOTOMICROGRAPHS TAKEN WITH 4 MM OBJECTIVE; MAGNIFICATION X 420, MALLORY-AZAN STAIN

1. Anterior pituitary of a normal control rat. Note the size, number and general distribution of acidophils. Note that the acidophils are conspicuous due to the deeply stained granulation which in many instances completely fills the cytoplasm.

2. Photograph showing the same region of the anterior pituitary of a rat injected for 437 days with growth hormone. Note the decrease in number and size of acidophils and loss of granular material. The majority of cells present are chromophobes.

granulation, and transformed into chromophobes. Nuclei were light staining and vesicular in type.

Figures 1 and 2 show typical sections through the pituitaries of injected and control animals. The general distribution, number, and size of the acidophils in the normal pituitary of a female 645 days of age can be seen in Figure 1. It will be noted that the cytoplasm is packed with deeply stained granules. Figure 2 shows the same region in the pituitary of an injected rat. The decreased size and the degranulation of the acidophils can be seen. The majority of the cells present are chromophobes.

Some of the acidophils were vacuolated. The vacuolation appeared to start in the cytoplasm, but at times also involved the nucleus. This process was observed in animals which had increased greatly in body weight as the result of growth hormone injection, but did not occur in all injected rats; it was not seen in the pituitaries of uninjected rats.

*Basophils*

In the majority of the experimental animals (6 out of 8) the pituitary basophils were not visibly altered by the injections. There was no apparent numerical change; the size was slightly but not significantly increased. (In planimeter units the mean size of the basophils in these six injected rats was $340 \pm 11$, in the controls it was $319 \pm 9$, which is not statistically significant.) The basophils in the pituitaries of the remaining two injected rats were markedly modified. Many of the basophils in these two glands showed changes similar to those seen after castration. Both early and late castration changes were present. The number of basophils was obviously increased. The average size was increased 50% More than 20% of the basophils were large, round cells, resembling those seen soon after castration. Signet-ring forms constituted 30% of all basophils. Numerous small, round basophils were also present, indicating that a new crop of young basophils was developing from the chromophobe reserve. The negative image of the Golgi apparatus was increased in size in all the basophils. As these two animals which had castration changes in the basophils had atrophic ovaries, the pituitary changes might be assumed to be related to the ovarian changes. However, other animals in the group had non-cyclic ovaries which were atrophic, yet clear cut castration changes in the pituitary basophils were not seen. It should be noted that even in these two pituitaries, which

had marked changes in the basophils, the acidophils showed the same changes which characterized the remainder of the experimental animals.

No significant changes were found in the pars intermedia or in the posterior lobe of any pituitary in the experimental group.

## Summary

In conclusion, it may be said that the most consistent changes in the anterior pituitaries of rats injected chronically with pure growth hormone were in the acidophils. The changes in the acidophils were observed in all animals of the group. The acidophils were decreased in number and size with a conspicuously decreased content in granules. The chromophobes were increased in number.

A similar decrease in either number or size of the pituitary basophils did not occur; indeed, in two of the eight injected animals these cells were increased in number and size in the way characterizing the pituitary after castration.

As the acidophils are postulated to be the source of growth hormone, and as the function of endocrine organs is frequently depressed by the presence of an excess of their own hormonal principle, the main histological change in the pituitary as the result of chronic injection of growth hormone may be interpreted as a depression in the functional activity of the cells producing it.

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*Growth*, 1948, **12**, 39-42

# THE GIGANTISM PRODUCED IN NORMAL RATS BY INJECTION OF THE PITUITARY GROWTH HORMONE

## *III. Main Chemical Components of the Body*[1]

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Paper I of this series has shown that the anterior hypophyseal growth hormone induces continuous growth in normal adult rats. During the period of injection, 437 days, the average gain of the injected animals was 289 grams and that of the controls 59 grams. It will be seen in the following that this gain in body weight was true growth.

*Experimental.* The details of the conduct of the experiment have already been described (Evans, Simpson and Li, 1948). After autopsy, the skin was separated from the eviscerated carcass, and both were frozen immediately. The water content in the tissue was estimated from the weight loss after lyophilization. The fat was extracted thoroughly from the dried carcass and skin by means of petroleum-ether in a Soxhlet apparatus. The ether extract was evaporated and the residue was dried to constant weight. The weight of the dry residue was taken as the measure of the total fat in the tissue. The fat-free dried tissue was next pulverized in a ball mill. The nitrogen content of the powdered tissue was determined by micro-Kjeldahl, a factor of 6.25 being used for conversion into the protein value.[2]

*Results.* Table 1 shows the mean composition of the carcass and skin in 7 growth-hormone injected rats and in 5 controls. It will be seen that an increase in water and protein content and a decrease in fat occurred both in the carcass and the skin as the result of hormone

[1]Aided by grants from the American Cancer Society, Inc., Research Fund, New York City, the United States Public Health Service (RG-409), Washington, D. C., and the Board of Research of the University of California, and the Rockefeller Foundation, New York City.

[2]The technical assistance of R. Wilcox is gratefully acknowledged.

TABLE 1

COMPOSITION OF THE CARCASS AND SKIN AFTER CHRONIC INJECTION OF THE PITUITARY GROWTH HORMONE IN NORMAL ADULT FEMALE RATS

| Treatment | No. of rats | Body Weight Initial | Body Weight Final | Weight per 100 gm Carcass $H_2O$ | Fat | Protein | Ash | Weight per 100 gm Skin $H_2O$ | Fat | Protein | Ash |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | gm | gm | gm | gm | gm | gm | gm | gm | gm | gm |
| Injected[1] | 7 | 275 | 564 | 65.3 | 10.5 | 18.0 | 4.8 | 57.2 | 11.6 | 29.2 | 0.53 |
| | | ± 6.5[2] | ±27.8 | ±0.6 | ±0.9 | ±0.2 | ±0.2 | ±0.2 | ±1.4 | ±0.9 | ±0.01 |
| | | | | <0.001 | <0.001 | <0.001 | <0.05 | <0.001 | <0.001 | <0.02 | |
| Control | 5 | 244 | 301 | 58.2 | 19.8 | 15.0 | 5.7 | 47.1 | 24.3 | 25.8 | 0.52 |
| | | ±14.2 | ±12.8 | ±0.8 | ±1.3 | ±0.3 | ±0.1 | ±0.8 | ±1.1 | ±0.4 | ±0.01 |

[1]Growth hormone injected intraperitoneally, six days weekly for 437 days; daily dose 0.4 mg., 21 injections; 0.6 mg., 58 injections; 1.0 mg., 29 injections; 1.5 mg., 99 injections; 2.0 mg., 168 injections.

[2]The figures preceded by ± are the standard errors. The next row of figures gives the random probability (p) of difference between the injected group and the controls.

treatment. It was found by analyses of the data by the statistical method of Fisher that the differences in percentage composition of tissues between injected and control rats were all highly significant except in the case of the percentage of ash. The changes were more marked in the skin than in the carcass. In 100 gms of skin of the treated rats there was 11.6 gms of fat, 29.2 gms of protein and 57.2 gms of water, whereas the same amount of skin in the controls contained 24.3 gms of fat, 25.8 gms of protein and 47.1 gms of water. It is evident that the increase in body weight was not merely the result of accumulation of fat and water but that more protein was formed in the new tissue.

*Discussion.* The results just presented show that growth hormone causes a rise in protein and water content with a lowering of the fat content of the body. Similar results have been obtained by the injection of pituitary extracts which, though not purified, contained the growth hormone (2, 3, 6, 7, 9, 10). Since a higher content in protein and water and a lower fat content is characteristic of youthful tissues, and since these chemical changes are characteristic of normal growth (Moulton, 1923), it seems justifiable to call the increase in body weight induced by growth hormone, true growth.

It must be emphasized that the experimental animals reported herein were fed ad libitum. One might inquire as to whether the difference in the body composition between the injected and control groups could be related to the difference in food intake. However, the experiments of Lee and Shaffer (1934) and of Young (1945), in which the growth resulting from injection of anterior pituitary extracts was analyzed, have shown that the body composition of injected rats given food ad libitum, was qualitatively similar to that resulting when the food intake was limited.

Previous studies (1, 5) have shown that growth hormone induces a marked reduction of urinary nitrogen. From these results, together with the ones here reported, it may be concluded that growth hormone enhances the protein anabolic process. Since protein synthesis is generally believed to be a criterion of growth, the results herein reported, obtained with a hormone which induces accelerated growth, were to be anticipated.

## Summary

Chemical analysis has been made of the chief components of the bodies of rats which had received injections of growth hormone for a period of 437 days. The analyses showed an increased proportion of protein and water, and a decreased fat content in injected rats as compared with controls. These proportions are characteristic of the body during early, rapid, normal growth, and it would therefore seem that injection of the growth hormone gave tissues possessing the chemical constituents of the body in youth.

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*Growth*, 1948, **12**, 43-54

# THE GIGANTISM PRODUCED IN NORMAL RATS BY INJECTION OF THE PITUITARY GROWTH HORMONE

## *IV. Skeletal Changes: Tibia, Costochondral Junction, and Caudal Vertebrae*[1]

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The gigantism produced in normal rats as a result of chronic administration of growth hormone involved an increase in skeletal dimensions as well as a gain in body weight. General somatic growth has been described in the first paper of this series (Evans, Simpson, and Li, 1948). This and the succeeding papers are devoted to an analysis of the skeletal changes. The tibia, costochondral junction and caudal vertebrae will be considered here, and the succeeding paper will deal with the skull and dentition.

Eight normal female rats received injections of growth hormone for 14 months, starting when they were approximately 210 days of age, i.e. when the curve of growth had "plateaued". Four rats of the same age served as controls. All were maintained on a complete diet. Details concerning the care of the animals and the growth hormone preparation and dosage used are presented in the first paper in this series.

Periodic measurements of the length of the tail (anus to tail-tip) were made during the injection period. Body measurement (tip of nose to anus) and tail measurements were made under anesthesia at the termination of the experiment (Table 1). The animals were also photographed and roentgenographed at the end of the injection period.

At autopsy, the left tibias were dissected and measured with vernier

[1]Aided by grants from the American Cancer Society, Inc., Research Fund, New York City, the United States Public Health Service (RG-409), Washington, D. C., the Board of Research of the University of California, the Rockefeller Foundation, New York City, the American Foundation for Dental Science and the California State Dental Association.

TABLE 1

EFFECTS OF CHRONIC INJECTION OF GROWTH HORMONE ON SKELETAL DIMENSIONS OF NORMAL PLATEAUED FEMALE RATS

| Treatment | Animal number | Body Measurements Length at Autopsy: Nose-anus | Anus-tail tip | Total | Length of Caudal Vertebrae: 9th | 19th | Tibia: Length | Diameter of proximal epiphysis: Antero-Posterior | Lateral | Diameter of shaft at center | Width of proximal epiphyseal cartilage |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | cm | cm | cm | mm | mm | mm | mm | mm | mm | μ |
| Growth Hormone[1] | BH4012 | 23.0 | 20.0 | 43.0 | 10.9 | 7.8 | 42.8 | 6.3 | 8.0 | 3.4 | 171 |
| | GH2214 | 24.5 | 22.4 | 46.9 | — | — | 43.8 | 6.2 | 5.8 | 3.4 | 136 |
| | BH2342 | 24.8 | 21.1 | 45.9 | 10.1 | 7.9 | 43.9 | 6.5 | 7.8 | 3.6 | 122 |
| | G4058 | 24.4 | 21.0 | 45.4 | 11.0 | 7.6 | 45.3 | 7.2 | 7.2 | 3.8 | 144 |
| | B4064 | 23.4 | 20.3 | 43.7 | 10.0 | 7.6 | 45.2 | 6.2 | 7.7 | 3.6 | 171 |
| | W2166 | 24.6 | 19.8 | 44.4 | 9.8 | 6.2 | 43.4 | 7.0 | 8.5 | 3.8 | 169 |
| | BH4087 | 24.3 | 21.5 | 45.8 | 10.6 | 7.4 | 44.9 | 6.2 | 8.1 | 3.4 | 173 |
| | B4094 | 25.5 | 22.7 | 48.2 | 11.5 | 7.7 | 47.3 | 7.8 | 10.7 | 3.8 | 148 |
| | Mean:[2] | 24.3 ±0.28 | 21.1 ±0.28 | 45.4 ±0.60 | 10.6 ±0.25 | 7.5 ±0.20 | 44.6 ±0.30 | 6.7 ±0.21 | 8.3 ±0.35 | 3.6 ±0.07 | 154 ±6.90 |
| Control | BH2244 | 22.0 | 19.0 | 41.0 | 9.5 | 6.8 | 37.8 | 6.1 | 7.1 | 2.5 | 119 |
| | B2156 | 21.2 | 17.5 | 38.7 | 8.9 | 6.1 | 41.6 | 5.9 | 7.1 | 2.9 | 132 |
| | BH4077 | 21.3 | 18.2 | 39.5 | 9.0 | 6.4 | 39.1 | 5.9 | 7.5 | 2.7 | 132 |
| | B4093 | 23.0 | 19.9 | 42.9 | 9.8 | 6.9 | 39.9 | 6.2 | 7.5 | 2.9 | 136 |
| | Mean: | 21.9 ±0.42 | 18.7 ±0.52 | 40.5 ±0.93 | 9.3 ±0.21 | 6.6 ±0.19 | 39.6 ±0.76 | 6.0 ±0.08 | 7.3 ±0.12 | 2.8 ±0.10 | 130 ±3.48 |

[1]Growth hormone injected intraperitoneally, six days weekly for 437 days; daily dose gradually increased from 0.4 mg to 2 mg; average age of rats at autopsy 647 days.

[2]The means and their standard errors (figures preceded by ±) were computed with allowance for the small size of the samples, according to the formula, $\sqrt{\frac{N}{N-1}}$. Where desirable, the significance of differences between various means was tested by application of the formula $\sqrt{(\sigma m_1)^2+(\sigma m_2)^2}$. Where significant differences are claimed, the difference between the means exceeds this value by three or more times.

calipers. Several bones were fixed in neutral formol, again roentgenographed separately, and then decalcified. The specimens were embedded in nitrocellulose, sectioned, and stained with haematoxylin and eosin. The widths of the proximal epiphyseal cartilages of the tibias were measured with an ocular micrometer. Osteogenic and chondrogenic activity at the left proximal tibial epiphyses, at the ninth vertebral epiphyses of the tail and at the third left costochondral junctions were analyzed histologically.

The rats injected with growth hormone were significantly longer than the controls (45.4 as compared with 40.9 cm, Table 1); both trunk and tail contributed to this greater length, the proportion of tail length to body length not being changed by injection. The roentgenograms showed that the injected animals had larger skulls and greater size of thoracic cage, pelvis, vertebral column, tail and long bones. The fore and hind paws did not take part in these increases; this might have been anticipated, since epiphyseal union of the growth centers in the metacarpals, metatarsals, and phalanges had taken place before the beginning of the injections of growth hormone (Becks, Asling, Collins, Simpson and Evans, 1948).

## Tibia

The tibias of rats injected with growth hormone for 437 days were significantly longer than those of their corresponding controls, 44.6 mm compared with 39.7. Other dimensions of the tibias of experimental rats also increased. The antero-posterior diameter of the head of the tibia averaged 6.7 mm in the injected animals, a significant increase over the 6.0 mm diameter of the controls. The average of 8.3 mm for lateral diameter of the head may be considered significantly greater than the 7.3 mm diameter of the controls, although the test of significance gave a value of only 2.7. The diameter of the shaft at its midpoint was significantly greater in the injected group, 3.6 mm as compared with 2.8 mm in the controls. These differences in size may be seen in the roentgenograms of the tibias shown in Fig. 1. Figure 2 presents in graphic form the tibial lengths of animals in the two groups. It shows that in every case the rats in the injected group had tibias longer than those of their controls. The widths of the epiphyseal cartilages of the tibias are also shown in Figure 2. It will be seen that they are wider in injected animals than in controls.

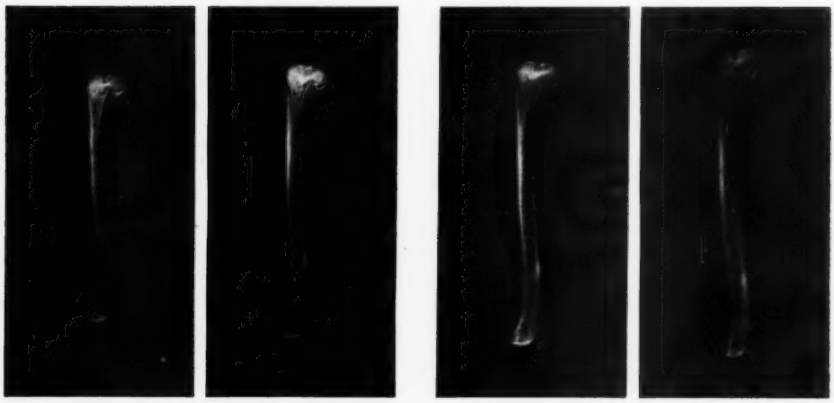


FIGURE 1

ROENTGENOGRAMS OF DISSECTED TIBIAS AFTER FORMOL FIXATION. NATURAL SIZE.
Left: Smallest and largest from control rats.
Sp. 8977 and 8278, Pl. 9625.
Right: Smallest and largest from growth hormone injected rats.
Sp. 8982 and 8989, Pl. 9626.

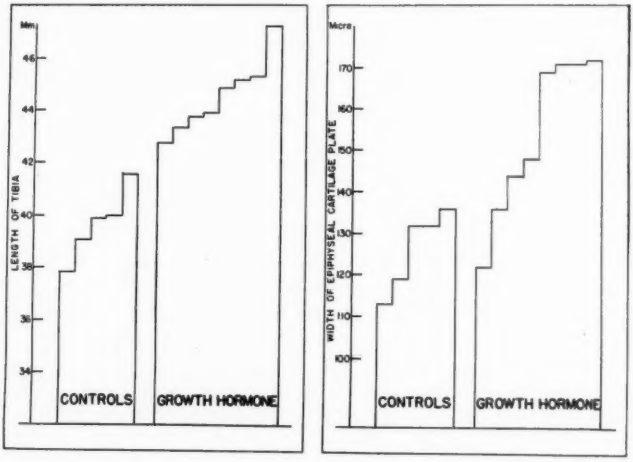


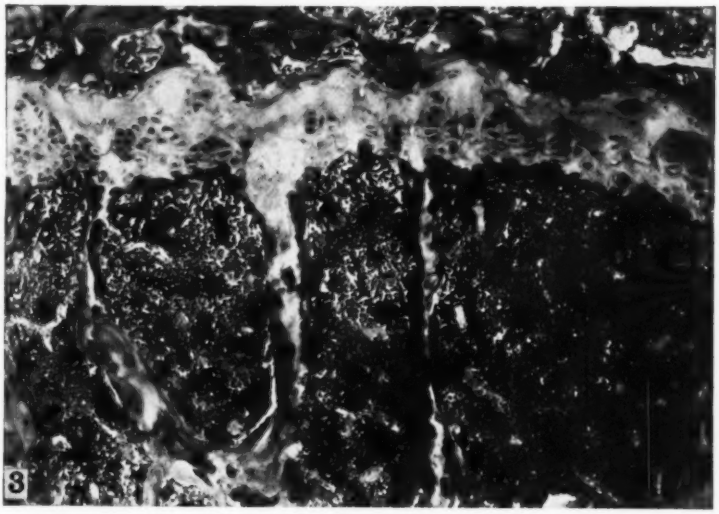

FIGURE 2

GRAPHS REPRESENTING MEASUREMENTS OF THE TIBIAS OF GROWTH HORMONE INJECTED AND CONTROL RATS. PL. 9627.

Left: Length in centimeters as measured on freshly dissected tibia with vernier calipers. There is no overlapping between the two groups.

Right: Width in micra of proximal epiphyseal cartilage as measured in histological preparations with an ocular micrometer. There is a slight overlapping between the groups but the difference between the means of the two groups is statistically significant.

PHOTOMICROGRAPHS OF MEDIAN SECTIONS OF THE PROXIMAL EPIPHYSEAL REGION OF THE TIBIA, 8-10 MICRA, H. AND E. X 112.



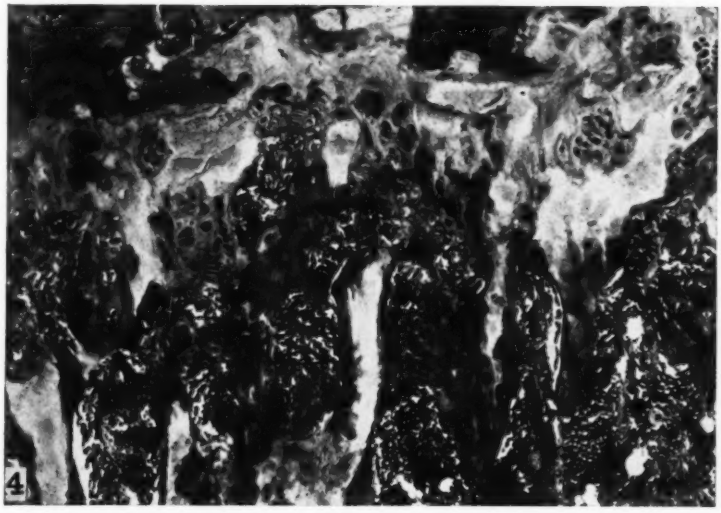




FIGURE 3
UNINJECTED CONTROL, 659 DAYS OF AGE.
Sp. 8978, Pl. 9654.

FIGURE 4
GROWTH HORMONE TREATED RAT (INJECTED FOR 437 DAYS PRIOR TO AUTOPSY AT 630 DAYS OF AGE). SP. 8988, PL. 9659.

The histological aspects of the region of the proximal epiphysis of the tibia are shown in Figures 3 and 4. This region in an uninjected control rat, 630 days of age, is shown in Figure 3. It will be seen that at this age the primary features of the epiphyseal plate are irregularity in width and general atrophy. The cartilage cells are small and frequently scattered, though some alignment is still recognizable. No true zone of enlarged, vacuolated cells is present. The relative amount of matrix is greatly increased; large areas stain lightly and contain only a few shrunken, pyknotic cells. Such changed, lightly staining matrix frequently extends through the width of the epiphyseal plate. A substantial layer of bone has "sealed" the cartilage from the marrow cavity of the diaphysis and capillary tufts rarely reach the cartilage. A few wide trabeculae of bone extend into the marrow cavity. Some of these contain a massive core of granular cartilage matrix which is continuous with similar masses in the cartilage plate. The over-lying bone on such trabeculae is thin, consisting sometimes of a single lamina of bone. Only a few flattened osteoblasts and rare osteoclasts are present on their surfaces. The cavity of the diaphysis is filled with a cellular marrow.

Figure 4 represents the epiphyseal region of the tibia of a rat injected with growth hormone for 437 days. A somewhat chaotic picture is presented. The cartilage plate is thin and no more regular in width than in the control. There are several features indicating increased activity. The cartilage cells are larger; many are arranged in definite rows. The relative amount of matrix is less than in the controls and it is more normal in appearance. It is more homogeneous and more normally staining. Masses of granular cartilage matrix are, however, present. No true layer of vacuolated cells exists, though groups of them are irregularly present next the marrow. There is an almost complete absence of "sealing-off bone", and vascular tufts impinge upon and are eroding the lacunar walls. These tufts may be so broad that they are in contact with 3 or 4 columns of enlarged cartilage cells. There is much variation in the extent of advancement of the capillaries, with consequent variability in cartilage thickness. As in the controls, there are coarse bony trabeculae enclosing considerable masses of cartilage. In addition there is a network of more delicate trabeculae. Both coarse and fine trabeculae are frequently covered by a single lamina of bone. Osteoblasts are present on the surface of the trabeculae and some of them are rounded instead of

flattened as in the controls. Osteoclasts are more easily distinguishable than in the controls. The marrow is cellular and moderately vascular.

Even though this picture offers evidence for active osteogenesis in the injected rats whereas there was none in the controls, the processes are slow. The slow rate of these changes is indicated by the absence of a well defined zone of vacuolated cartilage cells, the absence of capillary contact with each cartilage column (so that many trabeculae are coarse), the enclosure of masses of cartilage in the trabeculae, and the greater distance the trabeculae extend into the marrow, indicating lack of resorption and reorganization.[2]

In the distal end of the tibia of rats of this group no epiphyseal cartilage plate was found. This is consistent with the reported maturation of this epiphysis at the age of approximately 95 days. The linear growth of the tibia which resulted from the injection of the growth hormone must therefore have been due solely to activity at the proximal end of this bone.

### Costochondral Junction

The costochondral junctions in the uninjected rats present typical indications of senescence, in accord with their age of 629 to 658 days (Fig. 5). At the junction of the cartilage with the rib proper, there is found first a region of undifferentiated hyaline cartilage, showing small rounded cells imbedded in an abundant matrix. Next there appears an area of flattened chondrocytes, sometimes arranged in rows or cones. The intervening matrix, although abundant, is not as excessive nor as degenerate as that seen in the tibia. The vesicular zone in most cases is completely absent. There is very little evidence of capillary erosion of the cartilage. In most cases cellular elements of the marrow are separated from the cartilage by a layer of bone, which is thicker than that seen in the tibia. The marrow cavity is usually free of bony trabeculae; the few which are present are short and broad, and extension of cartilage into these is not as marked as in the tibia.

[2]In one of the eight injected rats, a perforation of the epiphyseal cartilage had taken place. A narrow bony union was established between epiphysis and diaphysis, and the cartilage plate was completely interrupted in the region of its anterior one-third. This may be worthy of note in view of the fact that maturation of the proximal end of the tibia and disappearance of its epiphyseal cartilage is stated by Dawson (1925), to occur much later (viz. at about 1135 days) in the normal rat.

PHOTOMICROGRAPHS OF SECTIONS OF THE COSTOCHONDRAL JUNCTION FROM LEFT THIRD RIBS, 8-10 MICRA, H. AND E. X 100.

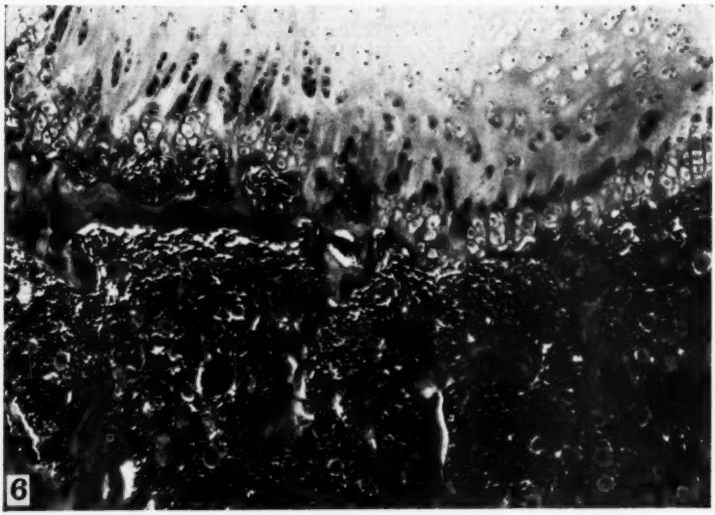
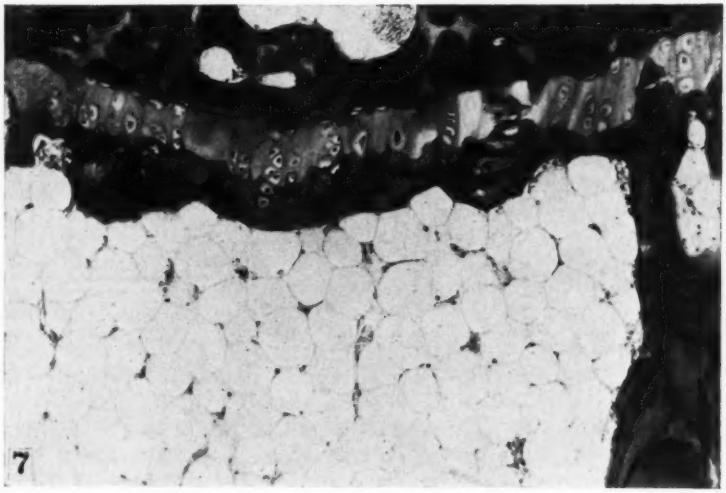



FIGURE 5
UNINJECTED CONTROL, 659 DAYS OF AGE.
Sp. 8978, Pl. 8973.

FIGURE 6
GROWTH HORMONE TREATED RAT (INJECTED FOR 437 DAYS PRIOR TO AUTOPSY AT 630 DAYS OF AGE). SP. 8985, PL. 8974.

PHOTOMICROGRAPHS OF MEDIAN SECTIONS OF THE NINTH CAUDAL VERTEBRA, 8-10 MICRA, H. AND E. X 123.



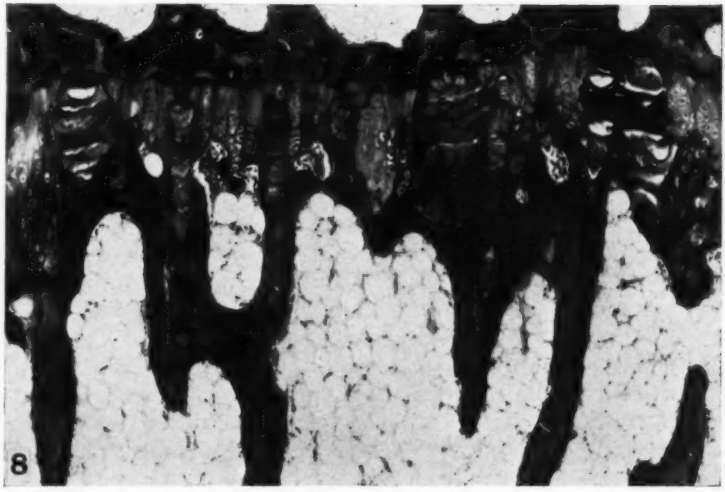




FIGURE 7
UNINJECTED CONTROL, 659 DAYS OF AGE. SP. 8978, PL. 9660.

FIGURE 8
GROWTH HORMONE TREATED RAT (INJECTED FOR 437 DAYS PRIOR TO AUTOPSY AT 630 DAYS OF AGE). SP. 8988, PL. 9662.

The injected rats invariably show costochondral junctions whose organization indicates an activity greater than that found in their controls (Fig. 6). Even the region of undifferentiated cartilage shows stimulated cell division. There is a decreased amount of matrix. The flattened, proliferating cartilage cells are arranged in moderately long columns. A zone of enlarged cells is always present, and there is definite vacuolation. These vesicular cells are largely exposed to capillary erosion. The trabeculae are increased in number, are longer and more delicate, and contain very little cartilage. The marrow is cellular.

### Caudal Vertebrae

The ninth and nineteenth caudal vertebrae were selected for measurement and histological study. In measurements made on roentgenograms of the tails, the ninth, the more proximal and larger of the two, had an average length of 9.3 mm in the controls and 10.6 mm in the injected animals. The nineteenth caudal vertebrae was 6.6 mm long in controls, and 7.5 mm in the injected rats.

Histologically, each vertebra shows a body separated at either end from the epiphyseal ossification centers by an epiphyseal cartilage plate. The marrow cavities of both epiphyses and diaphysis are filled with adipose tissue in all cases.

The epiphyseal region of the ninth caudal vertebra of control rats contains a cartilage plate which is narrow, and which has small cells scattered in an abundant matrix (Fig. 7). There is no zone of enlarged cells. The matrix is degenerate in many areas, where it consists of great vacuoles filled with coarse shreds of substance. The cartilage is sealed off from the fatty marrow by a substantial layer of bone. The cartilage matrix next the "sealing-off bone" is more basophilic than in the rest of the cartilage. This cartilage is continued into the center of some of the bony trabeculae. The trabeculae are straight and slender, and frequently join the network of cancellous bone which fills the center of the marrow cavity.

In the injected rats, the ninth vertebra shows only a few indications of activity greater than that of the controls (Fig. 8). The epiphyseal cartilage is no wider, but it contains cells which are increased in number. The cells show the usual tendency to alignment in rows. There are groups of such cellular columns, and other areas where none exist. Near the diaphysis some cells are enlarged, but are not

always vacuolated. The matrix is less basophilic than in the controls, but shows the same degenerate areas. Small islands of cellular marrow containing some capillaries are in contact with the cartilage, and little sealing-off bone is seen. The number of trabeculae is increased, and many lateral anastomoses occur between adjacent trabeculae, so that the marrow cavity near the cartilage is honey-combed with bone. So much enclosed cartilage is continuous into this mesh-work that it is difficult to determine the exact margin of the cartilage plate.

The nineteenth caudal vertebra of the control rats has an architecture not distinguishable from that of the more proximal bone, except for its smaller dimensions.

The nineteenth caudal vertebra of the injected rats is similar to the more proximal bone. The main differences lie in more sparse trabeculation and fewer signs of capillary erosion, with the presence of large fragments of sealing-off bone.

Even though the degree of osteogenetic activity stimulated in any single vertebral epiphysis was less than that seen in the tibia or at the costochondral junction, it should be noted that there are more than 50 epiphyseal centers of growth in the tail.

## Summary

The outstanding result of this experiment is that skeletal growth was still continuing, even though slowly, at the end of the 437 day period of growth hormone injection. This was shown histologically in the proximal epiphysis of the tibia by the persistence of some zonal arrangement within the cartilage plate, the presence of some capillary erosion, and the formation of delicate trabecular bone. The corresponding region in the controls showed marked senescent atrophy.

Growth hormone also stimulated continued activity of bone formation at the costochondral junction, and contributed to the increased dimensions of the thoracic cage.

The proximal and distal epiphyses of the caudal vertebrae studied (9th and 19th) were also more active in the growth hormone treated than in the control animals. The degree of osteogenesis was, however, less than that seen at the proximal end of the tibia or in the costochondral junction.

## References

1. Becks, H., Asling, C. W., Collins, D. A., Simpson, M. E., & Evans, H. M. 1948. *Anat. Rec.*, **100**, 577-591.
2. Dawson, A. B. 1925. Age order of epiphyseal union in the long bones of the albino rat. *Anat. Rec.*, **31**, 1-17.
3. Evans, H. M., Simpson, M. E., & Li, C. H. 1948. The gigantism produced in normal rats by injection of the pituitary growth hormone. I. Body growth and organ changes. *Growth*, **12**, 15-32.

*Growth*, 1948, **12**, 55-67

# THE GIGANTISM PRODUCED IN NORMAL RATS BY INJECTION OF THE PITUITARY GROWTH HORMONE

## *V. Skeletal Changes: Skull and Dentition*[1]

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The general body growth and organ changes in normal rats injected chronically with pure growth hormone have already been described (Evans, Simpson, and Li, 1948). The preceding report on skeletal changes dealt with the tibia, costochondral junction, and caudal vertebrae (Becks, Collins, Asling, Simpson, Li and Evans, 1948). The present paper is devoted to the changes in the skull and dentition, with special emphasis on the mandibular condyle, the calvarium, the central incisors, and the molars.

The rats used in these experiments were normal female rats of the Long-Evans strain which had received daily intraperitoneal injections of growth hormone from 7 months of age for approximately 14 months. The response of 8 injected as contrasted with 4 control rats is described. Information as to the diet, growth hormone preparation and dosage may be found in the initial paper of this series. During the injection period, the rate of eruption of the upper incisors was followed by weekly measurements.

Roentgenographic studies of the skull were made at time of autopsy.[2] For these studies the heads were divided sagittally and fixed

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[2]It is to be noted in interpreting the roentgenograms that the top of the cranium and the sellar portion of the sphenoid bone had been removed to obtain the pituitary gland for cytological studies.

ROENTGENOGRAMS OF SKULLS OF RATS. X 2.

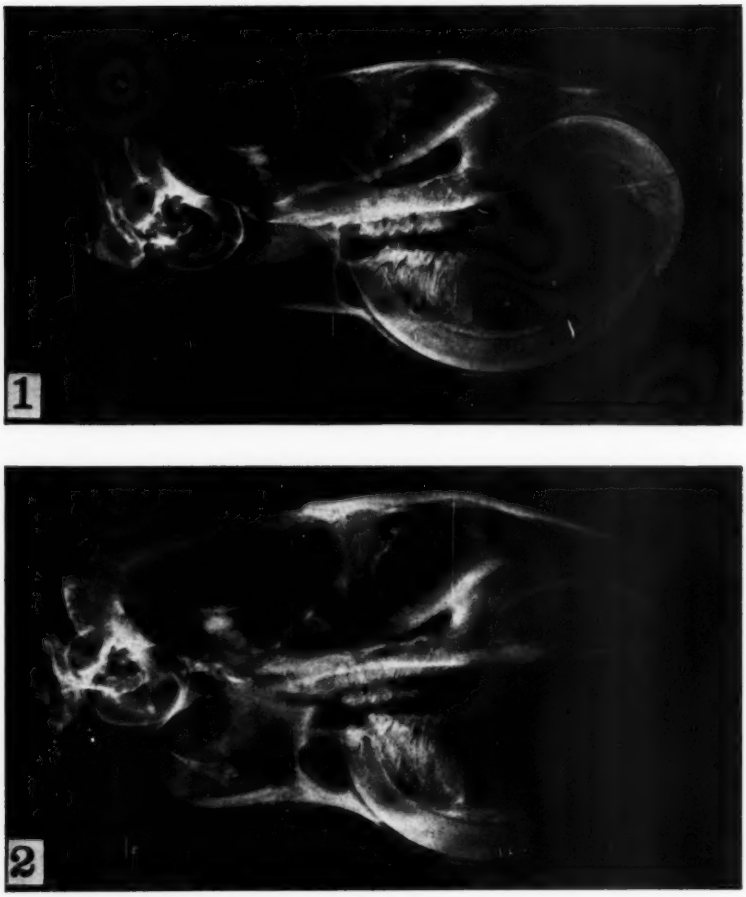


FIGURE 1
NORMAL 630 DAY OLD RAT. SP. 8979. PL. 7849.

FIGURE 2
GROWTH HORMONE TREATED RAT (INJECTED FOR 437 DAYS PRIOR TO AUTOPSY AT 630 DAYS OF AGE). SP. 8986, PL. 7849.

in neutral formol. For histological analysis half of the skull was decalcified, cut into blocks, imbedded in nitrocellulose, sectioned, and stained with hematoxylin and eosin.

## Skull: Maxilla and Mandible

Roentgenographic examination showed major alterations from normal in the skulls of the growth hormone treated rats (Figs. 1 and 2). The size of the skull was markedly increased; the sinuses were larger and the laminae of bone were thicker and denser. The increase in size of the teeth was shown especially in the incisors and in the length of the molar roots. Reference points for skull measurements were chosen in order to determine quantitatively whether the upper and lower jaw maintained the relative positions characteristic of the normal (Fig. 3). In the lower jaw the reference points for

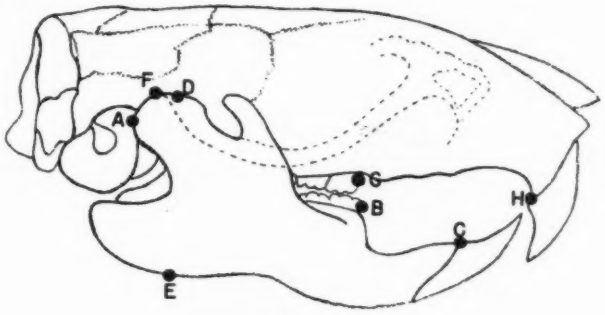


FIGURE 3
Diagram of Skull of Rat, Showing Reference Points for Measurements
A. Posterior border of mandibular condyle.
B. Mesial surface of 1st lower molar.
C. Lingual alveolar crest of mandible.
D. Superior surface of mandibular condyle.
E. Inferior border of ramus, vertically opposite to point D.
F. Posterior border of zygoma.
G. Mesial surface of 1st upper molar.
H. Lingual alveolar crest of maxilla.
Pl. 9628

measurements of the longitudinal dimensions were as follows: the posterior border of the mandibular condyle (A), the mesial surface of the 1st lower molar (B), and the lingual alveolar crest of the mandible (C). The reference points for measurement of the height of the mandible were the superior surface of the condyle (D), and

TABLE 1

EFFECT OF GROWTH HORMONE ON THE LONGITUDINAL AND VERTICAL MEASUREMENTS OF THE LOWER AND UPPER JAWS OF FEMALE RATS INJECTED FOR 437 DAYS, FROM 210 TO 647 DAYS OF AGE

| Measurements | | Reference Points | Control | Injected | Increase of Injected over Control Group | |
|---|---|---|---|---|---|---|
| | | | mm | mm | mm | % |
| Longitudinal | Lower Jaw | Posterior border of condyle to mesial surface of 1st lower molar (A - B) | 18.5 ± 0.23[1] | 21.6 ± 0.81 | 3.1 | 16.8 |
| | Upper Jaw | Posterior border of zygoma to mesial surface of 1st upper molar (F - G) | 17.2 ± 0.15 | 18.5 ± 0.23 | 1.3 | 7.5 |
| Longitudinal | Lower Jaw | Posterior border of condyle to lingual alveolar crest of mandible (A - C) | 26.1 ± 0.11 | 29.8 ± 0.31 | 3.7 | 14.2 |
| | Upper Jaw | Posterior border of zygoma to lingual alveolar crest of maxilla (F - H) | 30.8 ± 0.29 | 33.7 ± 0.29 | 2.9 | 9.4 |
| Vertical | Lower Jaw | Superior surface of condyle to inferior border of ramus of mandible (D - E) | 11.9 ± 0.15 | 14.1 ± 0.24 | 2.2 | 18.5 |

[1]Figures preceded by ± are the Standard Errors. All means derived from 4 control and 8 injected animals.

the inferior border of the ramus vertically opposite (E). In the upper jaw, the reference points for measurements of longitudinal dimensions were as follows: the posterior border of the zygoma (F), the mesial surface of the 1st upper molar (G), and the lingual alveolar crest of the maxilla (H).

As shown in Table 1, all longitudinal and vertical dimensions of both jaws were greater in the rats injected with growth hormone than in controls. The increase in the upper and lower jaws was, however, not the same. It will be noted from the table that the longitudinal measurement (A-B) of the lower jaws of the experimental animals exceeded those of controls by 3.1 mm whereas comparable measurements in the upper jaws (F-G) differed by only 1.3 mm. When the longitudinal measurements included the incisor segments of the alveolar bone, the measurements of the lower jaw of the experimental animals (A-C) exceeded those of controls by 3.5 mm, whereas comparable measurements in the upper jaw (F-H) differed by 3.2 mm. Following growth hormone injection there was, therefore, a disproportionately greater growth in length of the lower jaw than of the upper jaw. This difference in growth, however, was partially masked by inclusion of the incisor segments of the alveolar bone, indicating that the cause of the differential growth was to be sought in the posterior segment of the mandible. The vertical measurements of the mandible of injected rats (D-E, 14.1 mm) also exceeded those in controls (11.9 mm) by 2.2 mm. The greater growth of the mandible in injected rats was, therefore, manifested in height as well as in length. The degree of activity in the mandibular condyle resulting from injection of growth hormone may well account for both the horizontal and vertical growth in the lower jaw.

During active growth in young normal rats, the mandibular condyle shows four well-defined zones, as shown in the orientation photomicrograph, Fig. 4, and at higher magnification in Fig. 5. In the cartilage subjacent to the articular surface, there is first a zone of flattened germinal (embryonic) cells, whose long axis parallels the surface. Below these is the second or intermediate zone of rounded cells, whose outline is more distinct. The third, or vesicular, zone is characterized by enlarged cells whose cytoplasm is vacuolated, and whose nuclei are pyknotic and often hyperchromatic. In the fourth zone, the zone of erosion, many capillary tufts touch the cartilage, and erosion of the cartilage is active. The marrow cavity beneath is

SECTIONS THROUGH MANDIBULAR JOINT OF RATS. HEMATOXYLIN AND EOSIN. X 20 except as indicated.

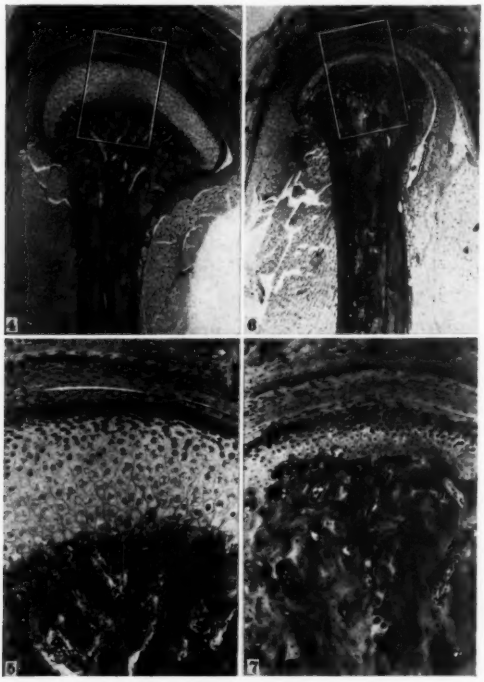

FIGURE 4
Normal, rapidly growing rat, 30 days of age. Sp. 8709. Pl. 9670.

FIGURE 5
Higher magnification (X 60) of the area outlined in Fig. 4.

FIGURE 6
Normal 195 day old rat, the age at which growth hormone injections were begun in the treated rats. Sp. 8376, Pl. 9648.

FIGURE 7
Higher magnification (X 60) of the area outlined in Fig. 6.

filled with a network of delicate bony trabeculae. The marrow is very vascular.

The histologic characteristics of the mandibular condyle at the onset of the growth hormone injections are represented by Figs. 6

SECTIONS THROUGH MANDIBULAR JOINTS OF RATS, SHOWING NORMAL CONTROL AT THE END OF THE EXPERIMENTAL PERIOD (645 DAYS OF AGE) AND VARIATIONS IN RESPONSE OF DIFFERENT RATS TO GROWTH HORMONE INJECTIONS. HEMATOXYLIN AND EOSIN. X 20.

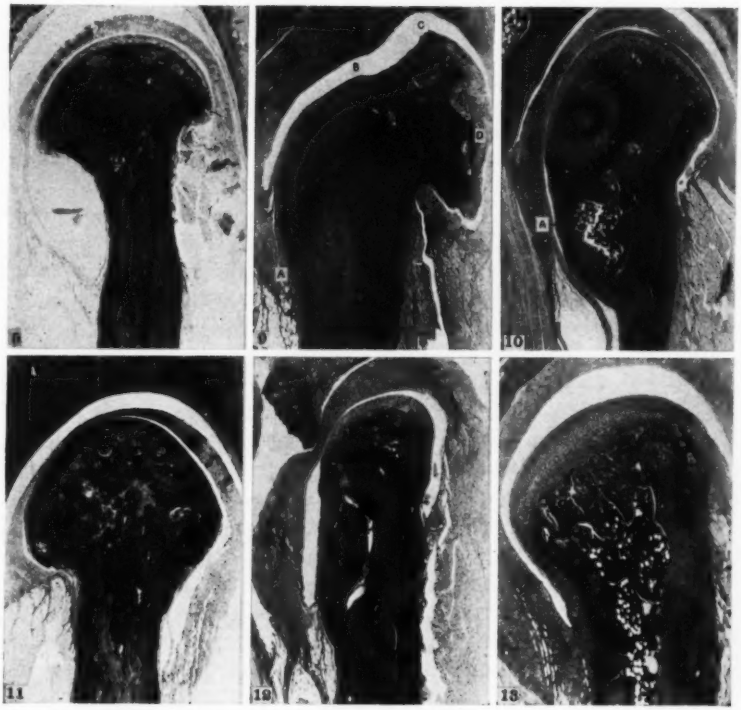


FIGURE 8
Normal rat. Sp. 8978 Pl. 9675.

FIGURE 9
Injected rat. Sp. 8989 Pl. 9238.

FIGURE 10
Injected rat. Sp. 8982 Pl. 9237.

FIGURE 11
Injected rat. Sp. 8985 Pl. 9198.

FIGURE 12
Injected rat. Sp. 8988 Pl. 9051.

FIGURE 13
Injected rat. Sp. 8987 Pl. 9191.

and 7, which show this growth center in a rat 195 days of age. By this time, when growth has virtually ceased, there remains of the cartilage only the zone of germinal cells, and a few small rounded cells indicative of the intermediate zone. No vesicular zone is left, and capillary tufts are sparse. The ramus of the condyle is composed of dense bone; only rare islands of marrow are seen. Further changes during the period covered by the injections are limited to a gradual encroachment of bone on the intermediate zone of the cartilage, until in the old normal rat the continuity of this layer is lost, as shown in the autopsy control (Fig. 8).

The mandibular condyles of rats which received growth hormone are represented in Figs. 9 to 13. It will be seen that the growth of the condylar head did not follow the normal pattern; in fact, grotesque distortions resulted. As the extent of these distortions varied, the condyles of five injected rats are illustrated. The changes in the other three injected animals followed the patterns shown in Figs. 10 to 12.

In Fig. 9 the regular arrangement of the compact bone of the ramus is interrupted along a sharply defined oblique line. Such lines are frequently found in older condylar heads, and apparently represent structural adaptations to functional stress. The orderly apposition of lamellar bone from this line to the surface at A is the result of periosteal ossification, and is continuing, as shown by the uninterrupted row of rounded osteoblasts in this region. The germinal (embryonic) zone of the cartilage, so prominent in the normal condyle, appears somewhat less cellular between B and C, and chondrocytes are almost entirely absent between C and D. The condylar head has lost its typical contour, with a resultant angulation and disfiguration. Areas of marked osseous resorption are present, many osteoclasts being found between C and D, lying below the acellular cartilage. The resulting bony surface is very irregular in outline. It is uncertain whether the disfiguration is a direct result of the administration of growth hormone or an adaptation to the changed functional requirements due to the disproportionate growth of the mandible.

Fig. 10 demonstrates another type of distortion of the condyle following chronic injection with growth hormone. A marked bulge containing a large marrow cavity is found on one side of the ramus at A. With the exception of this, it is possible to trace the original outline of the condylar neck and head. There is an increase in number and size of marrow spaces, especially at the border line between the

bone and the cartilage. In contrast to Fig. 9 the covering cartilage has a uniform germinal layer, which may account for the greater regularity of form of this condyle.

Fig. 11 is remarkable because of the inclusion of islands of cartilage lying deep in the compact bone of the condylar head. A few marrow islands are seen lying against the surface cartilage (as was also true in Fig. 10). The contour of the condyle is symmetrical, and except for its markedly greater size, resembles in outline the form of the normal control seen in Fig. 8. The germinal layer of the cartilage is as densely cellular and evenly distributed as in the normal control.

The condyle shown in Fig. 12 has elongated greatly at the expense of its characteristic shape. Resorptive activity can be seen on the lateral surface. Marrow cavities and vascular spaces are enlarged. The cellularity of the cartilage is reduced (see also Fig. 9).

Only one of the 8 animals which received injections of growth hormone for 14 months showed a cartilage whose histologic features were still characteristic of normal growth (Fig. 13). It resembled the condyle of a normal rat approximately 100 days of age, judged by the rate of chondrogenesis, the presence of some zonal arrangement of the cartilage, enlargement of the chondrocytes, erosion, osteogenic activity, and large size of the marrow cavities (Collins, Becks, Simpson and Evans, 1946). However, it is far larger than normal.

## Calvarium

*Study of the calvarium showed that the formation of membrane bone was stimulated by growth hormone.* Figure 14 shows a frontal section through the mid-point of the sagittal suture of a normal rat. This suture between the parietal bones is composed of fibrous connective tissue. The endocranium may be distinguished from the epicranium by the presence of the superior sagittal sinus. The inner and outer tables of bone are separated by diploic spaces containing hematopoietic marrow. The bony lamellae stain uniformly with eosin, with the exception of a few dark basophilic lines seen most prominently in the serrated bone opposing the suture. Active osteoblasts and osteoclasts are not seen, indicating that bone formation and remodeling were occurring slowly if at all.

Fig. 15 shows a corresponding section of the calvarium of a rat which had received growth hormone for 14 months. The thickness

FRONTAL SECTIONS THROUGH MID-POINT OF SAGITTAL SUTURE, BETWEEN PARIETAL BONES OF RATS. HEMATOXYLIN AND EOSIN. X 112.

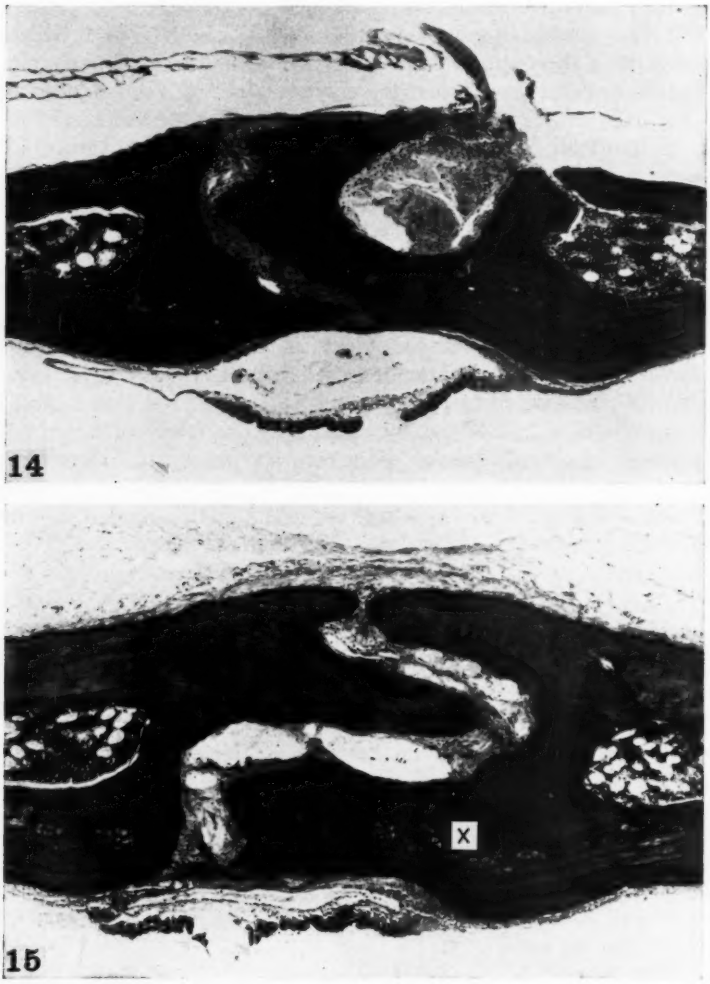


FIGURE 14
Normal 630 day old rat. Sp. 8981, Pl. 9189.

FIGURE 15
Growth hormone treated rat (injected for 437 days prior to autopsy at 630 days of age). Sp. 8982, Pl. 9188.

SAGITTAL SECTIONS THROUGH DISTAL ROOT OF LOWER FIRST MOLAR OF RATS.
HEMATOXYLIN AND EOSIN. X 57.

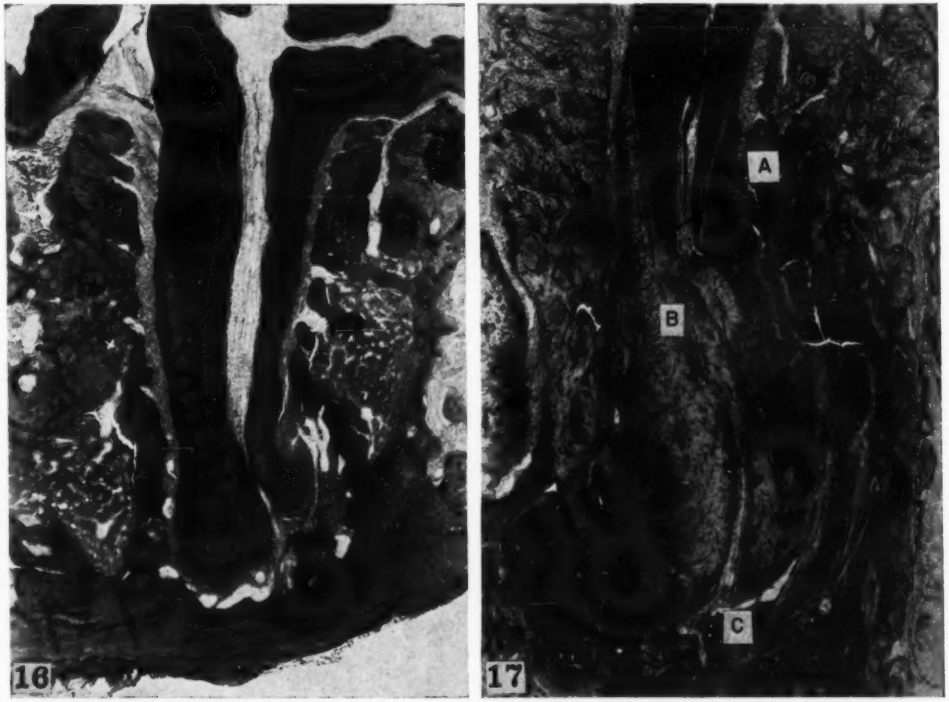


FIGURE 16
Normal 630 day old rat. Sp. 8979, Pl. 9672.

FIGURE 17
Growth hormone treated rat (injected for 437 days prior to autopsy at 658 days of age).
Sp. 8983, Pl. 9671.

of the parietal bone is far greater than in the controls.[3] The increased number of basophilic laminae shows the appositional growth which resulted in the increased thickness of the bone. Rounded osteoblasts were, however, rarely seen. The bone of the calvarium in injected rats showed certain imperfections. Many of the lamellae stained poorly with eosin. Numerous vacuoles were present in the bone of both inner and outer tables. Fibrous remnants could be seen between these confluent vacuolar areas (at X).

## Teeth

The incisors of the injected rats showed ameloblastic activity which extended nearly to the exposed surface of the teeth; in the normal controls the ameloblasts had degenerated in the central portion of the buccal surface. The odontoblasts likewise showed far less degenerative change in the incisal portion of the pulp tissue in injected rats than in the normal rats. The layer of dentinoid tissue was nearly twice as wide as in the controls. Measurement of the eruption rate for a six week period did not reveal any acceleration of eruption of the incisors resulting from the injections (*2.3 mm* per week in injected rats; *2.4 mm* per week in normal controls).

In the molars, the elongation of the roots which had been observed in roentgenograms of the skull of injected rats, was due to an excessive development of cementum (hypercementosis). Fig. 16 shows a sagittal section of the distal root of the lower first molar of a control rat, with a normal amount of cementum formation about the root. There are no indications of active cementogenesis. In marked contrast, the entire periphery of the area of hypercementosis of the injected rat (Fig. 17) bears a continuous row of active cementoblasts, separated from the cementum only by a very narrow layer of cementoid substance. Enclosed cementocytes (at A) are more numerous in the portion of the original apex (at B); more proximally the broad mass of the newer, lighter-staining cementum is less cellular. The blood vascular and nervous supply of the root thus elongated now enter at C. The paradental osseous structures of injected rats appear almost sclerotic, with very few open marrow spaces.

[3]At the suture, the average thickness of the calvaria of the injected rats was 846 micra, while that of controls was 529 micra. Lateral to the suture at a distance of 1 low-power field diameter, the measurements for injected and control rats were 660 micra and 379 micra respectively.

## Summary

Roentgenographic studies have shown that a generalized increase in size of the rat skull resulted from injection of growth hormone for 14 months. The growth of the mandible was, however, disproportionate, as it increased in size much more than did the rest of the skull. Histologically, the mandibular condyle showed a marked response. This resulted in enlargement and in most cases in distortion. In only one of the eight rats was the enlargement accompanied by the architecture which usually characterizes the normal growth of the condyle.

In the calvarium, stimulation of intramembranous bone formation took place. The history of the overgrowth of parietal bone could be seen in the series of lines of accretion.

The dentine and enamel-forming organs of the incisor teeth were stimulated by the growth hormone, but the result of this stimulation was not detectable by any increase in the rate of eruption of these teeth. There occurred an elongation of the molar roots, especially of the lower jaw, due to hypercementosis.

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# RETARDATION OF GROWTH AND METABOLISM OF NORMAL AND MALIGNANT CELLS DURING CONTINUOUS CULTIVATION[1]

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Throughout the life of multicellular organisms most of the tissues and organs are capable of carrying on their specific function with a fairly stable cell population and therefore at a low maintenance rate of growth. It is reasonable to assume that the ability of most adult cells to remain in long periods of growth dormancy is due to the fact that their host has provided them with optimum maintenance conditions through their specific environment. Great variations in the functional activities are not necessarily followed by loss of this growth dormancy.

When cells are released from the organization and control of the host and are implanted in tissue cultures, conditions are usually provided which cause them to migrate and divide rapidly. The present methods of maintenance *in vitro* in general are judged to be satisfactory if they provide for growth at nearly maximal rates, such as may occur in embryonic life or perhaps temporarily during repair or regeneration. The actual rate of tissue increase in a growing animal corresponds in fact to an almost imperceptible rate of growth in tissue cultures.

Since a major portion of biological and medical interest in the results of tissue cultivation depend on interpretation in terms of post-embryonic or adult physiology and pathology, it is obvious that the art and science of tissue cultivation need some re-orientation in the direction of maintaining more stable populations of cells which are not so exclusively concerned with rapid multiplication. Maintaining cells

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at metabolic levels which approximate those of post-partum physiology should be of value in studying problems concerned with cytology, nutrition, endocrine secretion, antibody formation, the inter-relations of cells and infectious agents, and the riddle of differentiation and malignancy. Such means should also aid greatly in the perpetuation of stock strains of cells over long periods of time with minimal effort.

Slow growth in the absence of embryo juice was shown by Fischer and Parker (5, 6) and Parker (23) to enable cells *in vitro* to carry on specific functions which were submerged during rapid growth. The role of heparin as a possible growth inhibitor has been the subject of a tissue culture study by Zakrzewski (36). Somewhat slowed growth was obtained by des Ligneris (17) in a serum embryo extract medium. Gey has shown (9) that in fluid cultures with only serum as a medium, reduction of the serum concentration to low levels allows cells to be kept in a fairly dormant state for long periods of time. Such means make possible a comparison of the metabolic activity of normal and malignant cells in the inter-mitotic period.

The use of reduced temperatures to keep cells at low metabolic levels was first reported by Lake (15), who demonstrated that —6° C. must be considered critical in relationship to the effects of cold upon tissues; and that cells, whether vegetative or actively growing, could be stored at +2° C. up to sixteen days with rapid growth occurring when re-incubated at 37.5° C. Fischer (4) was able to store cells of warm-blooded animals for a month or more, while Nemoto (22) was able to increase both total growth and longevity of chick cultures at reduced temperatures. As was shown in the work of Lake, Mayer (19) has shown that cold storage (+9.5° C. for 24 hours) is completely reversible. Bucciante (2) showed that the rate of cell division varies considerably with temperature and that mitoses still proceed to finish at 24° C. Lower temperatures resulted in a standstill of growth. Sano and Smith (27, 28) showed reduction of temperature to be compatible with wound healing and to permit slow growth and maintenance of normal and malignant cells for as long as six months. Spear (33) has also studied the influence of reduced temperatures on rates of mitosis in tissue cultures. Hanks (12) was able to demonstrate survival of chick tissues cultures for periods of three or four months and in this case without renewal of the medium.

The lowering of cell metabolism at reduced temperatures has been reported by Pinkerton and Hass (24) and by Sanders and Molloy (20, 25, 26) to facilitate the propagation of Rickettsial and virus agents in tissue cultures. The observations by Hanks (13) on enhanced destruction of leprosy bacilli in naturally infected human tissue cultures at the higher metabolic levels suggest the importance of quiescent cell cultures as a basic condition which might permit *in vitro* growth of the leprosy bacillus. The importance of adaptation to temperature, and the function of time, have been studied by Mast and Prosser (18) and by Hopkins (14) for the amoeba in studies on locomotion and other cell activities. The present study has not specifically taken into account the rate of change of temperature. The temperature variations imposed required at least several hours for completion.

In order to more closely approximate post-partum physiology by *in vitro* methods, one has a choice of trying to supply nutrients at the slow and continuous rates which obtain *in vivo,* or the other alternative of periodically supplying the excess nutrients which contribute to the usual rapid growth of tissue cultures. Although the periodic application of nutrients at lowered temperature was not regarded as the ideal way to control growth *in vitro,* this alternative was chosen in the present study because of its practical aspects.

## Methods and Materials

The general method of cell maintenance was based on that described by Gey and Gey (10). From one to four explants or subcultures (average 1.0 cu. mm. each) were imbedded on the walls of 16 x 150 mm. pyrex tubes, the plasma film being composed of two drops (approximately 0.12 ml.) of heparinized (1:250,000 Lederle synthetic) chicken plasma and coagulated by three drops (approximately 0.18 ml.) of the medium being used as a liquid phase. After orientation of the fragments of tissue about one-half of the mixture was discarded before coagulation occurred. The liquid phase was supplied as ten drops (approximately 0.6 ml.) of a mixture of human placental serum and beef embryo juice in the balanced salt solution. The embryo extract was prepared by homogenizing equal weights of beef embryo and balanced salt solution in a sterile Whiz-Mix[1] for

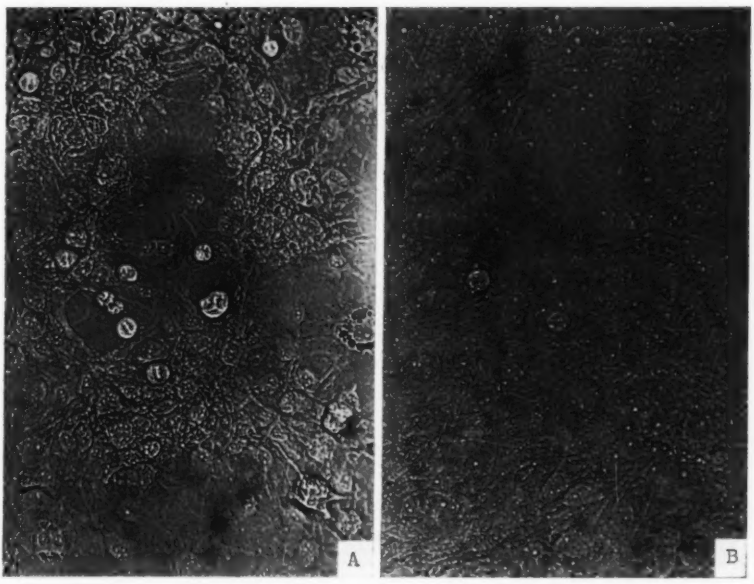
[1]A mechanical blender similar to the Waring Blender.

one to two minutes. This suspension was clarified by strong centrifugation two times. A compact cotton pledget was used as a sliding filter in the second centrifugation. The extract was then stored in the deep-freeze at approximately —40° C. Composition of the media will be designated as percentage concentration of serum (S) and embryo extract (Em), $S_{50}Em_5$ for example, representing 50% serum and a 1:10 dilution of the 50% embryo extract.

Incubation was carried out in four incubators equipped with roller drums and maintained at temperatures of 37° C., 34° C, 31° C., and 28° C., respectively. The results at reduced temperatures were assessed by several different criteria, the most important of which were: the presence of cells of normal appearance, persistence of mitosis, the ability of colonies to be transplanted continuously during slow growth, or the demonstrated recovery of active growth at 37° C. The majority of the experiments at low temperature were designed to investigate the maintenance of cell colonies by "renewal" or "transfer" at pre-selected intervals, by "renewal or transfer as required", and by "maximal survival without renewal". The effect of varying the composition of the medium, or of differing numbers of colonies per tube were studied in some instances. The "renew as required" schedules were based either on renewal when dividing cells became difficult to find or on the pH changes observed in media containing 0.002% phenol red (non toxic brand) indicator. In general, these several methods can be evaluated as follows. Fixed schedules of renewal do not apply the medium at rates proportional to the cell population to be maintained, but they are procedures honored by wide usage and, once established, are practical in routine work. Though observation of the pH change does not permit distinguishing between the production of organic acids and the rate at which they are consumed or at which carbon dioxide is released, it does provide an estimate of the over-all metabolism. Once one determines for any given strain of cells how far the pH may be allowed to fall or, if the acid is neutralized by sodium bicarbonate, how many times it may fall over a restricted range, it permits replacement of the medium as required by the increasing cell population. The criterion of renewing only when dividing cells become very rare leads to a greater exhaustion of the medium than do fixed schedules or the pH method, for dividing cells frequently persist in the marginal areas of colonies long after the cell mass as a whole is becoming definitely handicapped.

## Cell Strains

Many types of cells have been isolated in our tissue culture laboratory, and some have been maintained in continuous cultures for many years. The living cultures, for example Walker Rat Sarcoma 319 (Fig. 1), if grown in thin clot, show cells that are usually spread




2733—37° C. 2743—28° C.

FIGURE 1

Living single cell-layer spread of Walker Rat Sarcoma 319. Strain over 15 years in continuous culture. (A) Edge of roller tube control colony showing ten dividing cells. Grown on schedule of weekly renewal at 37° C. Photo at six weeks. (B) Same strain on schedule with renewal once in three weeks at 28° C. Two dividing cells present. Photo at four weeks.

out in a single layer over a broad surface which allows rapid and adequate diffusion of substances into and out of the cells. All of the strains used in this study showed in general this thin type of outgrowth, and often with a single layer of cells at each surface interface with the medium.

*Autologous Rat Strains*

*Normal Strain 14pf* was derived from the subcutaneous areolar tissue of a young adult rat (inbred strain) on October 1, 1938, and has been kept in pure strain to date (8.5 years). The thin, delicate cells vary morphologically from fusiform to polygonal types and form grossly an almost transparent colony. In stock media ($S_{66}Em_8$) at 37° C., it has a moderate rate of growth.

*Malignant Strain 333* arose during continuous culture from strain 14pf when it was over two years old. The spontaneous conversion occurred in one of several parallel lines. This pair of strains, 14pf and 333, have provided us with a normal and a malignant strain of autologous origin which are ideal for comparative studies. The malignant cells are larger and thicker than the normal strain of origin and show greater variation in size of cells and mode of division. Colony outgrowth is fairly compact and usually a single layer of cells with a grossly visible colony margin. The growth rate is rapid.

*Altered Normal Strain AN 21* arose about four months after primary explantation of the same tissue fragments from which normal strain 14pf was derived (Gey 11; Firor and Gey 3). Inoculation of this strain has resulted in the production of sarcomas which differ in their cultural and cytological characteristics.* This altered normal strain shows cells of irregular spindle type which vary greatly in size. The cells are thicker than the autologous normal 14 pf and the malignant 333. Binucleated and multinucleated cells are found frequently, and some of these divide into two or several daughter cells, commonly of unequal size. Colony outgrowth is compact and usually shows a single layer of cells at the fluid and at the glass interface. It has a grossly visible irregular colony margin. Growth is fairly rapid.

*Homologous Rat Sarcoma Strains*

*Walker Rat Sarcoma 319 and the Emge Rat Sarcoma* were used. The Walker Rat Sarcoma 319 has been maintained in continuous culture over 15 years, and its cultural characteristics are fairly well known (Schopper 29; Lewis 16; Gemmill, Gey and Austrian 8). For the most part when transplanted in thin plasma films, the outgrowth is a single cell layer composed of multipolar cells with numerous,

*To be published.

fairly long, delicate processes. The cells usually appear to lie apart from one another in strain cultures and a cohesive colony is maintained with some difficulty. Colony outgrowth is grossly only fairly well seen. Growth is moderately rapid.

The Emge Rat Sarcoma has been maintained in continuous culture for several years. It was made available to us through the courtesy of Dr. Charles M. Pomerat. When transplanted in thin plasma films, the outgrowth is compact with cells in single layers for each interface and usually only one layer at the outer margin. The sarcoma cells are fairly thin and vary morphologically from spindle to somewhat polygonal types. Dividing cells are very numerous and differ greatly in size. The colony outgrowth is grossly only fairly well seen. Growth is rapid.

## Results

Of the reduced temperatures studied, 31° C. proved in general to be the most practical for those strains which tolerated the usual medium, $S_{66}Em_8$. The growth rates at 34° C., though slower than at 37° C., proved to be too similar to be of special interest. At 31° C. growth was depressed more sharply but not too severely. At 28° C., with the methods and media used, growth tended to be extremely slow and continuous cultivation for some strains presented more serious problems.

### *Normal Rat Fibroblasts No. 14pf*

The Normal Rat Strain No. 14pf could be carried in $S_{66}Em_8$ at either 37° C. or 31° C. with one or four colonies per tube. Reference to Table 1 reveals that at 37° C. with one and four colonies per tube treatment once a week provides for adequate maintenance. At a two-week schedule, however, the cultures were lost from causes other than restricted feeding, and have therefore been replaced with a new series. The results, not yet reportable, suggest adequate maintenance. The four-week interval was obviously too great, although the health of the cultures was good up to three weeks. Beyond this, they rapidly declined, indicating that perhaps at this temperature cultures might be maintained for some time at a three-week treatment schedule with a transfer each time. These cultures have been set up and are still being studied.

At 31° C. four colonies per tube survived four weeks per renewal.

TABLE 1
PRELIMINARY RESULTS ON STRAIN NO. 14pf IN $S_{60}Em_5$
(Set up August 23, 1946)

| Renewal every | Maintenance of Cultures (to 26.IV.47) | | Transfer interval in weeks | Drops of medium per colony per week |
|---|---|---|---|---|
| | 1 colony per tube | 4 colonies per tube | | |
| **37° C.** | | | | |
| 1 week | Successful | Successful | 3 to 4 | 2.5 |
| 2 weeks | Unsuccessful[1] (1) | Unsuccessful[1] (2) | 4 | |
| 4 weeks | Unsuccessful[1] (2) | Unsuccessful[2] | 4 | |
| **31° C.** | | | | |
| | Maintenance of Cultures (to 26.IV.47) | | | |
| 1 week | Unsuccessful[1] (4) | Successful | 3 to 4 | 2.5 |
| 2 weeks | Unsuccessful[1] (1, 3, 4) | Successful | 4 to 8 | 1.25 |
| 4 weeks | Successful[3] | Successful[3] | 4 to 8 | 0.6 |

[1]Continuation of cultures interrupted by (1) contamination, (2) liquefaction of medium, (3) poor transfer, (4) the initial transfer of this particular group of cultures at 31° C. was made at 6 weeks which handicapped these cultures when they were continued on the scheduled transfer interval of 3 to 4 weeks and finally resulted in unsuccessful maintenance.

[2]Culture history showed abrupt decline in health after three weeks. Interval obviously too long.

[3]Up to seven months. Much poorer condition thereafter.

It was, in fact, practical to extend the transfer interval to twice that regarded as standard in this experiment. With only one colony per tube, the more frequent renewals were not advantageous, and possibly undesirable, considering the variations arising from the use of chicken plasma and cord serum selected at random. It would appear from the data that maintenance at 31° C. required approximately one-fourth as much medium as at 37° C. and that this amounted to only 0.6 drop of medium per colony per week when colonies were transferred before reaching a maximal size. It is interesting to remark that at 37° C. colonies achieved spreads up to 15 mm. where they remained constant, the only change noticed thereafter being an increase in cell size and granularity. Evidently, the mitotic interval was greatly increased, since many dividing cells were present for several weeks with no marked increase in colony size.

*Malignant Rat Fibroblasts, No. 333*

A preliminary experiment covering a few schedules for temperatures from 37° C. down to 28° C. is shown in Table 2 and deals only

TABLE 2

PRELIMINARY RESULTS ON STRAIN 333 IN $S_{66}Em_8$ AND FEASIBILITY OF SCHEDULES FOR MAINTENANCE

| | 37° C. | | | 34° C. | | |
|---|---|---|---|---|---|---|
| Renewal every | Maintenance of Cultures (to 26.IV.47) 3 col.*/tube[1] | 4 col./tube[2] | Transfer interval in weeks | Maintenance of Cultures (to 26.IV.47) 3 col./tube[1] | 4 col./tube[2] | Transfer interval in weeks |
| 0.5 week‡ | Successful | Successful | 1.5 | Successful | | 3 |
| 1 week | | Unsuccessful | 2 | Successful | | 2 to 3 |
| 1.5 week | | | | | | |
| 2 weeks | | | | | | |
| 3 weeks | | | | | | |
| 4 weeks | | | | | | |
| MINIMAL QUANTITY OF MEDIUM PER COLONY PER WEEK | | | | | | |
| 0.5 week‡ | | 5 drops | | | | |
| 1 week | | | | | 2.5 drops | |
| 1.5 week | | | | | | |
| 2 weeks | | | | | | |
| 3 weeks | | | | | | |
| 4 weeks | | | | | | |

| | 31° C. | | | 28° C. | | |
|---|---|---|---|---|---|---|
| Renewal every | Maintenance of Cultures (to 26.IV.47) 3 col./tube[1] | 4 col./tube[2] | Transfer interval in weeks | Maintenance of Cultures (to 26.IV.47) 3 col./tube[1] | 4 col./tube[2] | Transfer interval in weeks |
| 0.5 week‡ | Successful | | 3 to 4 | Successful (?)[3] | | |
| 1 week | | | | | Unsuccessful | 3 |
| 1.5 week | Successful | | 4.5 | | | |
| 2 weeks | | Successful | 4 | Unsuccessful | Unsuccessful | 4 |
| 3 weeks | | Successful | 6 | Successful[3] | Unsuccessful | 3 (also 6) |
| 4 weeks | | Successful | 4 | | | |
| MINIMAL QUANTITY OF MEDIUM PER COLONY PER WEEK | | | | | | |
| 0.5 week‡ | | | | 5 drops | | |
| 1 week | | | | | | |
| 1.5 week | 2.2 drops | | | | | |
| 2 weeks | | 1.25 drops | | | | |
| 3 weeks | | 0.83 drop | | 0.83 drop | | |
| 4 weeks | | 0.60 drop | | | | |

‡Actually 3 da. followed by 4 da. and repeat.

*Colonies.

[1]Set up January 23, 1946.

[2]Set up October 23, 1946.

[3]This culture remained at 28° C. for 3.5 weeks and was then returned to 37° C. for 1 week at which time there was a new outgrowth of active cells emigrating from brown debris and areas of large granular giant cells. It was then put back at 28° C. for 15.5 weeks at which time there were no recognizable emigrating living cells. A return to 37° C. for 7 weeks effected a gradual but complete recovery. The culture was then put at 31° C. and remained in good condition for 7.5 weeks. It was then put at 28° C. for 3 weeks. At this time it was sub-cultured to make 7 tubes for a new experiment at 28° C. This was a bold experiment because the colonies were cut up into tiny fragments whose further survival was thereby impaired. One of the more successful cultures of this group was maintained further at 28° C. for 9 weeks with transfers every 3 weeks and with no intervening treatment. Two successful transfers using one-fourth of the culture on this schedule was possible. A third required the use of all of the culture. After this, the schedule was altered so as to include a patch at 3 weeks and a transfer at 6 weeks. At the time of the second transfer on this new schedule, there were two 3.5 mm. colonies with medium to fairly large broad spindle and polygonal cells of granular appearance. This, then, was the condition of the culture after 24 weeks at 28° C. The culture was then incubated at 37° C., but the results thus far indicate that recovery is unlikely.

with cultures having three and four colonies per tube. The results indicate that this strain could be carried in $S_{66}Em_8$ on a treatment schedule of two times per week when incubated at 37° C., 34° C., 31° C., or 28° C., with the transfer interval progressively increased to accommodate the decrease in growth rate which accompanied the progressive decrease in temperature. Since this was a preliminary experiment, it could not be stated that the transfer intervals which were successful actually represent the optimal period for transfer for any given temperature. In fact, in attempting to maintain cultures at 28° C. on this treatment schedule of two times per week, the need for transfer had to be judged by the appearance of the cells, since colony growth was very slow. When the cells showed many brown granules, there was always a tendency to attempt to improve their condition by changing the treatment schedule. An example of considerable variation in the treatment of one of the cultures at 28° C. appears in the footnote (3) of Table 2 which covers a maintenance period of 24 weeks at 28° C., but with a return to 37° C. twice in the interval, once after 3.5 weeks at 28° C. and a second time after 15.5 weeks at 28° C. The re-activation of cultures at 37° C. may be considered an important criterion for viability when used on cultures that appear to be inactive. This criterion was successfully used by Nemoto (22). The results on this culture suggest very long maintenance at 28° C., with a return to 37° C. at infrequent intervals. This seems to be necessary for re-activation of stalled cultures. Regarding the renewal schedule, we are inclined to believe that these cultures were fed much too frequently.

On a treatment schedule of one time per week with four colonies per tube at 37°C. and a transfer interval of two weeks, good maintenance was possible. A transfer and treatment schedule of one time per week is feasible but more laborious. At 34° C. with three colonies per tube and a renewal once a week, a transfer interval of two to three weeks provided adequate maintenance. At 31° C. with three colonies per tube, the treatment interval could be extended to 1.5 weeks with transfers every 4.5 weeks. In fact at the same temperature, successful maintenance was possible with treatment intervals of two, three, and four weeks and with transfers at four, six, and four weeks, respectively. At 28° C. with treatment at intervals, varying from one treatment per week to one treatment in four weeks,

the results showed unsuccessful maintenance over this range of renewal schedules, with the exception of the culture referred to above which was successfully maintained for a long period at 28° C. on a three-week renewal schedule and with transfer at three and at six weeks.

As stated previously, the results at 31° C. demonstrate good growth repression of about the same degree whether renewal occurs two times per week or as infrequently as once every four weeks, the transfer interval being about four weeks in each case. In most of the cultures that we have run at this temperature level, the cells appear to be in good condition.

*Tumor Producing Altered Normal Rat Fibroblasts, No. 21*

The success of cultivation of this strain of cells at low temperature was variable, like that of Walker Rat Sarcoma No. 319, being dependent on several conditions. The most important of these was to start with stock material entirely free of cells with brown granular cytoplasm, to imbed the colonies in moderately thin plasma films, and to use media in which the glucose levels were at 120 mg. per cent or lower. As an example of the unsatisfactory results which follow upon the use of stock material with a modest proportion of brown granular cells, one experiment is reported. Table 3 and Figure 2 summarize the number and proportion of colonies remaining in good condition in each of three temperature groups after two and

TABLE 3

STRAIN AN-21

Proportion of good colonies remaining after two and four weeks of maintenance at 34° C., 31° C., and 28° C. in $S_{85}M_6$, glucose 120 mg.%.

| Temperature | No. of tubes | Good colonies after 2 weeks | | Good colonies after 4 weeks | |
|---|---|---|---|---|---|
| | | Ratio | Per cent | Ratio | Per cent |
| 34° C. | 15 | 42/45 | 94% | 39/45 | 87% |
| 31° C. | 17 | 48/51 | 94% | 36/51 | 71% |
| 28° C. | 19 | 51/57 | 90% | 14/54* | 26% |

*One tube excluded due to wet stopper.

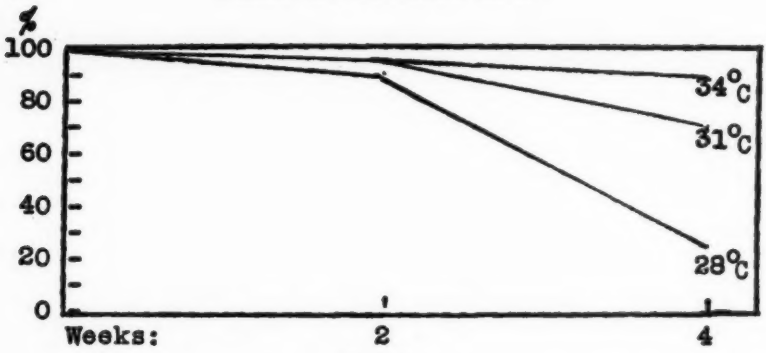



FIGURE 2
STRAIN AN-21
Proportion of useful colonies after two and four weeks at 34° C., 31° C. and 28° C.

four weeks. It may be seen that the results at the different temperatures fell in an orderly sequence, and that 28° C. was distinctly less favorable than 31° C. or 34° C. In spite of attempts to improve the condition of the colonies by renewal of some at four weeks, while still at low temperature, their quality continued to decline with an associated accumulation of brown cytoplasmic granules. Recoveries at 37° C., therefore, were undertaken after five and six weeks and later. Six weeks was the longest interval after which recoveries were possible from cultures of the 34° C. and 31° C. sub-groups, while 28° C. cultures failed to recover after this interval. The most suitable method of recovering such colonies was to add to the used medium about one-half its volume of new medium, to place the tubes at 37° C., and to renew several times in sequence as required. Transplantation as individual colonies was not successful until after a partial recovery of the retarded colonies had been achieved. Thereafter, the return to normal growth rates and transplantability were rapid.

In attempts to circumvent these difficulties, it was learned that colonies in the standard medium remained in better condition if the medium was not renewed. Increased glucose (0.2%) was much more unfavorable than the usual levels (0.12%), while one-half this amount (0.06%) produced colonies which improved during a period of one month and were successfully transferred to a further series of tubes. Reduction of the embryo juice from 6% to 3% was also found advantageous. Further observations on the effects of still lower concentrations of nutrients are indicated.

In experiments with cells which initially were devoid of the brown granular cytoplasm, it was shown that renewals at either two- or three-week intervals were satisfactory and permitted transfer at intervals of six to twelve weeks (see Table 4). After maintenance at 31° C. for twelve weeks, most of the cells had acquired brown granules, but were continuing to grow. At 28° C. this strain survived for only six to ten weeks on the treatment schedules used above.

As may be seen in the table, the requirements at 37° C. were successfully reduced to one-eighth the amount of medium which has been used routinely over a period of over eight years for maintenance of stock cultures. Cultures carried at 31° C. required a minimum of only 0.83 drop per colony per week or one-twelfth the medium used for stock maintenance at 37° C.

*Walker Rat Sarcoma, No. 319*

The Walker Rat Sarcoma proved much less adaptable to growth at low temperature. The difficulties encountered here were attributed, at least in some measure, to liquefaction of the plasma clot, which usually had necessitated patching at each renewal (two times per week) and a transfer every ten to fourteen days and to the resultant loose colony architecture when the cultures in these experiments were patched less frequently. The individual cells in this strain were severely handicapped when growth was repressed and the cells existed without the benefit of close proximity to others. Although 25% serum was more favorable than 66%, none of the media or renewal schedules permitted much more than one serial transfer at 31° C. or 28° C. (Figures 3 and 4). In fact, the culture groups designed to be renewed only when required by the disappearance of dividing cells (Figure 5) survived longer than those groups in which the medium was renewed more frequently. In non-renewed media ($S_{25}$ or $S_{66}$), it required on the average ten weeks for the disappearance of dividing cells. Following renewal after such intervals, a small and delayed wave of mitosis appeared in about one and one-half weeks, but further renewals did not stimulate renewed cell division. This is the first example of a strain which can withstand quite long intervals of growth repression, but evidently cannot be maintained continuously without alternate periods of growth activation.

TABLE 4

PRELIMINARY RESULTS ON STRAIN AN-21 IN $S_{\infty}Em_{8}$ AND FEASIBILITY OF SCHEDULES FOR MAINTENANCE*

| Renewal every | Maintenance of Cultures | | Renewal interval in weeks | | Minimal Quantity Medium/col./week | | Cell Condition at 12 weeks |
|---|---|---|---|---|---|---|---|
| | 1 col.‡/tube | 4 col./tube | Transfer | Patch | 1 col./tube | 4 col./tube | |
| | | | 37° C. | | | | |
| 1 week | Successful | Successful | 2 | 1 | 10.0 drops | 2.5 drops | Slightly granular |
| 1.5 weeks | Feasible | Successful | 1.5 | 1.5 | | 1.66 drops | " |
| 1.5 weeks | Successful | Unsuccessful (?)[1] | 3 | 1.5 | 6.6 drops | | " |
| 2 weeks | " | Successful | 2 | | 5.0 drops | 1.25 drops | " |
| | | | 31° C. | | | | |
| 2 weeks | Feasible | Successful | 6 | 2 | | 1.25 drops | Granular |
| 2 weeks | " | " | 12 | 2 | | 1.25 drops | " |
| 3 weeks | " | " | 6 | 3 | | 0.83 drop | " |
| 3 weeks | " | " | 9 | 3 | | 0.83 drop | " |
| 3 weeks | " | " | 12 | 3 | | 0.83 drop | " |
| | | | 28° C. | | | | |
| 2 weeks | | Unsuccessful | | | | | Dead at: 10 weeks |
| 3 weeks | | " | 6 | 3 | | | 6 weeks |
| 4 weeks | | " | 8 | 4 | | | 10 weeks |

*Transferred into medium clot and patched with thin clot.
‡Colonies.
[1]Successful maintenance probably obtained with care.

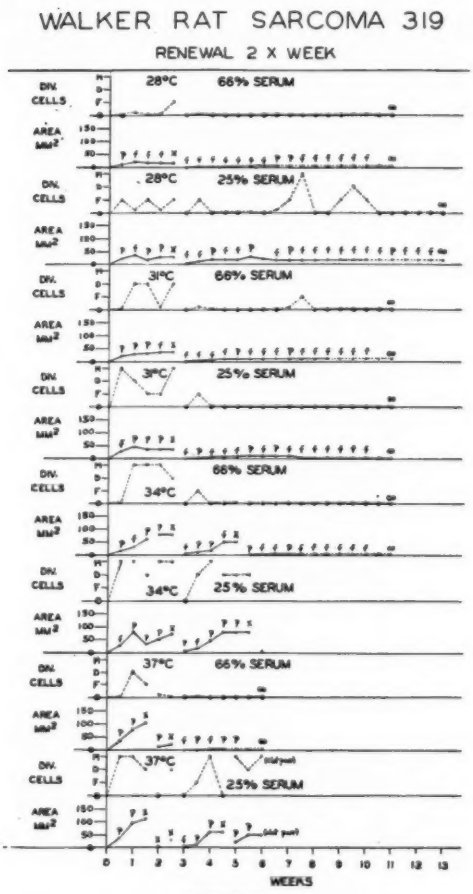



FIGURE 3

Shows area of colonies and record of dividing cells at different temperatures with treatment twice per week.

Dividing cells: M=many; D=moderate number; F=few

Renewal: P=patch; f=fluid; x=transfer; ∞=death of colony

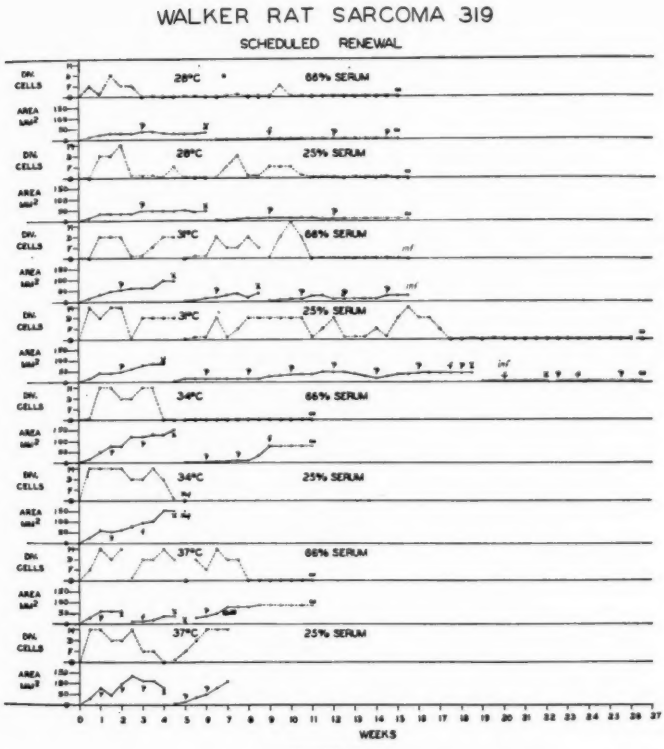



FIGURE 4

Shows prolongation of treatment schedules with reduction of temperature. Reduction in size of colonies at 28° C. is shown. Delayed mitotic waves following treatment apparent at low temperatures.

Dividing cells: M=many; D=moderate number; F=few

Renewal: P=patch; f=fluid; x=transfer; ∞=death of colony

*Emge Rat Sarcoma*

Of all strains employed, this one showed at all times the greatest number of dividing cells. On scheduled renewals of $S_{66}Em_3$ twice per week at 37° C., this strain was thought to require transfers approximately every ten to fourteen days to maintain active healthy cells. When fed according to this schedule at 31° C., a preliminary experiment showed the transfer interval could be extended to four

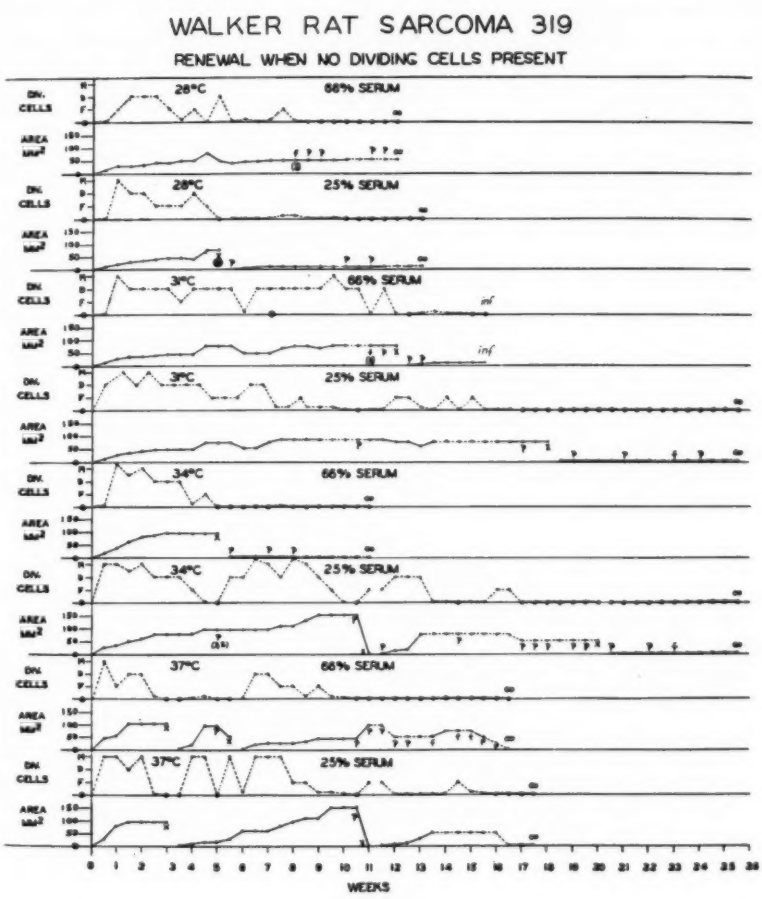



FIGURE 5

Shows poor results following attempted transfer of exhausted cultures. Results at 31° C. shows some promise of continued maintenance with more favorable treatment. First mitotic waves represent carry-over from previous 37° C. maintenance.

Dividing cells: M=many; D=moderate number; F=few

Renewal: P=patch; f=fluid; x=transfer; ∞=death of colony

and one-half weeks, with dividing cells constantly present (Figure 6). At 28° C. dividing cells persisted throughout transfer intervals averaging seven weeks, or even as long as eleven weeks. The growth rates at 34° C. were somewhat lower than at 37° C. Growth at 31° C. was much more markedly repressed, to about one-fourth the rate at 37° C., while growth at 28° C. was almost negligible. In spite of almost stable cell populations at the two lower temperatures, there was an unexpected proportion of dividing cells over periods lasting many weeks. This suggests that cells remained in mitosis for long periods of time before completing division.

In another experiment renewal of the medium was deliberately delayed until required by the absence of dividing cells (Figure 7). At 37° C. mitotic cells were not seen after three to four weeks. When colony fragments were transferred to new medium after these intervals (three to four weeks), a new wave of mitosis reappeared, and then rapidly subsided after about ten days, reaching low levels within two weeks, and tapering off again to no dividing cells by the end of three to four more weeks. This cycle was repeated after each transfer. At 34° C. dividing cells usually disappeared by the end of five weeks, and transfers were required. Over a period of six months, the wave of mitosis following each transfer was consistently more prolonged than at 37° C. At 31° C. without renewal, some dividing cells persisted for periods averaging seven weeks. Following transfer, the mitotic wave at first persisted for about four weeks before the sharp decline, but later the waves became less marked and less persistent as the cells approached six months of maintenance on a minimal feeding and transfer schedule. At 28° C. there was a carry-over of active mitosis which must be attributed to the fact that the cells were derived from 37° C. cultures. Within three and one-half weeks, mitosis declined until it was decided to transfer the colonies. Following this transfer, no wave of mitosis appeared and growth was negligible, and the usual wave of mitosis was delayed for five weeks. This wave was small and promptly subsided. Since no mitotic cells were found within the next week, the question of treatment was solved by replacing one-half the liquid phase, and this caused a very slight wave of mitosis after a delay of one and one-half weeks. A second interim application of completely renewed fluid again required one and one-half weeks to cause recognizable but transitory mitosis.

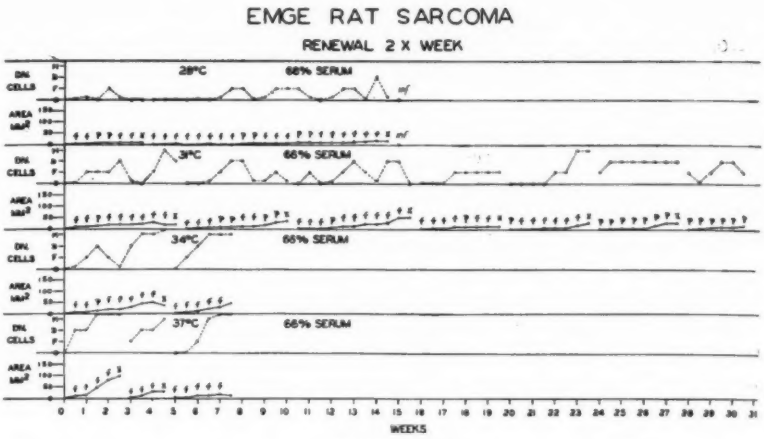



FIGURE 6

Shows marked retardation of growth at 28° C. and 31° C. with an unexpected proportion of dividing cells.

Dividing cells: M=many; D=moderate number; F=few

Renewal: P=patch; f=fluid; x=transfer

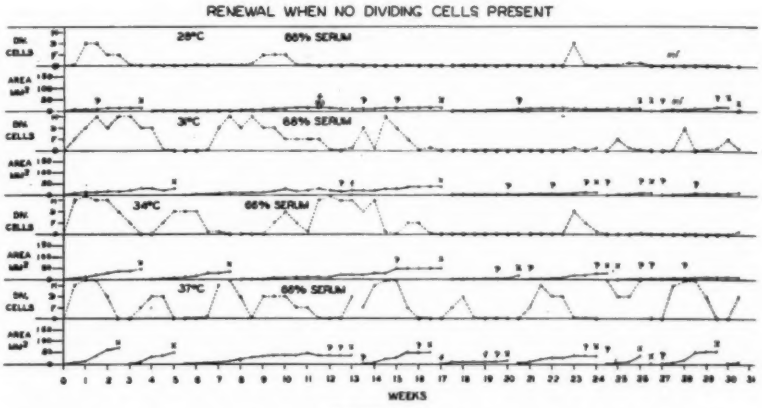



FIGURE 7

Shows waves of mitosis following renewal or transfer with progressively greater delay of mitotic waves accompanying reduction of temperature.

Dividing cells: M=many; D=moderate number; F=few

Renewal: P=patch; f=fluid; x=transfer

Following a subsequent transfer, there was a similar delay before mitosis occurred to an appreciable extent.

The contrasting results in these two experiments reveal that when nutrients were applied at frequent intervals (two times per week), mitotic cells are usually present at all temperatures, and that low temperature was chiefly responsible for retarding growth. When, on the other hand, the nutrients were withheld, waves of mitosis occurred only subsequent to transfer or feeding. Under these circumstances, there was at 28° C. a delay before the effect of the new nutrients became manifest in mitosis. Analysis of such results by Willmer's method (35) of serial photomicrography would be very desirable.

Further experiments (Table 5) dealing with different renewal and transfer schedules at 37° C., 31° C. and 28° C. revealed that three weeks between transfers at 37° C. was the longest period during which continuous growth could be relied upon in serial cultivation. At 31° C. a schedule of four weeks between renewals and eight to sixteen weeks between transfer was successful. At 28° C. intervals of eight weeks between renewals and eight to sixteen weeks between transfer proved successful.

Thus the minimal requirements of these cultures of sarcoma for temperature and nutrient did not differ greatly from those of Normal Strain No. 14pf at 37° C. and 31° C. Even though the number of visible mitoses was always greater in these tumor cells, it did not grow more rapidly than the Normal Strain 14pf. The results imply that at the reduced temperature, it has a longer period in mitosis.

## Fibroblasts from Adult Human Skin

### *Influence of Temperature on Primary Explants*

Thiersch graft fragments (1.5 × 1.5 mm.) from a patient 55 years of age were explanted in a thin composite plasma clot with $S_{66}Em_6$ and incubated at 31° C. and 37° C. The medium was renewed whenever the pH fell to approximately 7.2. As may be seen in Table 6 and Figure 8, the proportion of explants which became positive for epithelial cells increased in the two groups at comparable rates during the first week. At 37° C. good epithelial growth was transient, while at the lower temperature the proportion of explants with epithelial cells increased steadily during the first four weeks.

The proportion of primary explants which became positive for

TABLE 5

PRELIMINARY RESULTS ON EMGE RAT SARCOMA IN $S_{88}Em_2$ AND FEASIBILITY OF SCHEDULES

| | 37° C. | | | | 31° C. | | | | 28° C. | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Renewal every | Maintenance of Cultures (to 1.V.47) 4 col.*/tube[1] | Renewal interval in weeks Transfer | Patch | Minimal Quantity Medium/col./week | Maintenance of Cultures (to 1.V.47) 4 col./tube[1] | Renewal interval in weeks Transfer | Patch | Minimal Quantity Medium/col./week | Maintenance of Cultures (to 1.V.47) 4 col./tube[1] | Renewal interval in weeks Transfer | Patch | Minimal Quantity Medium/col./week |
| 1 week | Successful | 2 | 1 | 2.50 drops | Successful | 10 | 2 | 1.25 drops | | | | |
| 2 weeks | Successful | 2 | | 1.25 drops | | | | | Successful | 12 | 2 | 1.25 drops |
| 2 weeks | Unsuccessful | 4 | 2 | | | | | | | | | |
| 3 weeks | Successful | 3 | | 0.83 drop | | | | | | | | |
| 3 weeks | Unsuccessful | 6 | 3 | | Successful | 8 | 4 | 0.62 drop | | | | |
| 4 weeks | Unsuccessful | 4 | | | Successful | 16 | 4 | 0.62 drop | | | | |
| 4 weeks | | | | | Unsuccessful | | 6[2] | | | | | |
| 6 weeks | | | | | | | | | Successful | 12 | 6 | 0.42 drop |
| 8 weeks | | | | | | | | | Successful | 8 | 8 | 0.31 drop |
| 8 weeks | | | | | | | | | Successful | 16 | 8 | 0.31 drop |

*Colonies.
[1]Transferred into medium clot and patched with thin clot.
[2]Run for 11.5 weeks only.

TABLE 6
PROPORTION OF EXPLANTS SHOWING GROWTH OF EPITHELIUM AND OF FIBROBLASTS DURING PRIMARY OUTGROWTH AT 31° C. AND 37° C.

| Growth period (weeks) | Proportion of explants positive for: Epithelium 31°C | | Epithelium 37°C | | Fibroblasts 31°C | | Fibroblasts 37°C | | Total Positive Explants 31°C | | Total Positive Explants 37°C | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 11/23 | 48% | 8/17 | 47% | 2/23 | 9% | 3/17 | 2% | 13/23 | 57% | 11/17 | 65% |
| 2 | 17/23 | 74% | 9/17 | 53% | 8/23 | 35% | 8/17 | 47% | 18/23 | 78% | 11/17 | 65% |
| 4 | 19/23 | 83% | 5/14* | 36% | 17/23 | 74% | 10/14 | 72% | 20/23 | 87% | 10/14 | 72% |

*Number of explants decreased due to late development of contamination in one tube.

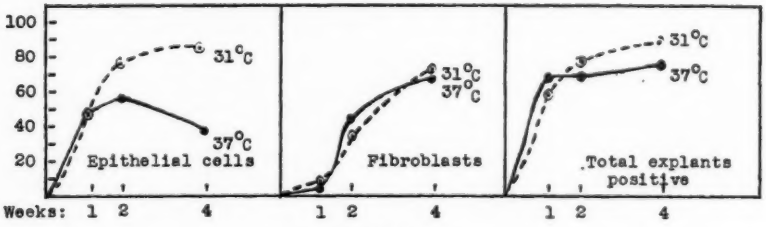



FIGURE 8
THE PROPORTION OF EXPLANTS PRODUCING EPITHELIAL CELLS AND FIBROBLASTS, AND OF TOTAL EXPLANTS POSITIVE, DURING PRIMARY GROWTH FROM HUMAN SKIN AT 31° C. AND 37° C.

fibroblasts was comparable in both the low and the higher temperature groups, though the growth rates were very dissimilar, and the fibroblasts at the lower temperatures had not yet produced an extensive outgrowth by the end of four weeks when the colonies at the higher temperatures required a transfer.

These results have since been confirmed, and they show that the lower temperature provided the epithelial cells with a relatively advantageous circumstance. The cultures at low temperature produced four colonies of epithelial cells in which fibroblasts failed to appear. These pure epithelial cell growths were maintained in the original tubes at 31° C. temperature for nearly six months before they were discarded. Attempts to transfer serially the epithelial cells from human skin and to maintain them in a state of constant proliferation

were not successful. A single colony of fibroblasts was maintained in one of the original tubes at 31° C. for seven months on a "renewal as required" basis.

*Influence of Temperature on Transferred Cultures*

Fragments from 37° C. cultures were used to compare the growth rates and the utilization of medium at 31° C., 34° C., and 37° C. Six tubes with three colonies each were carried at each temperature, the liquid phase being ten drops of $S_{40}Em_4$. The principle adopted was to renew the medium on the cultures at 31° C. after intervals of three, two, and one weeks (total six weeks before transfer), at 34° C. after two and one weeks (total three weeks), and at 37° C. after intervals of one week, four days, and three days (total two weeks) before transfer. By measuring the fall in pH at each interval, it was possible to learn whether corrections in the feeding rate should be made.

The newly transferred colonies (Figure 9) depressed the pH of the medium to pH 7.3 within three weeks at 31° C., two weeks at 34° C., and one week at 37° C., in good agreement with the anticipated needs. The cultures at 37° C. continued to utilize the medium

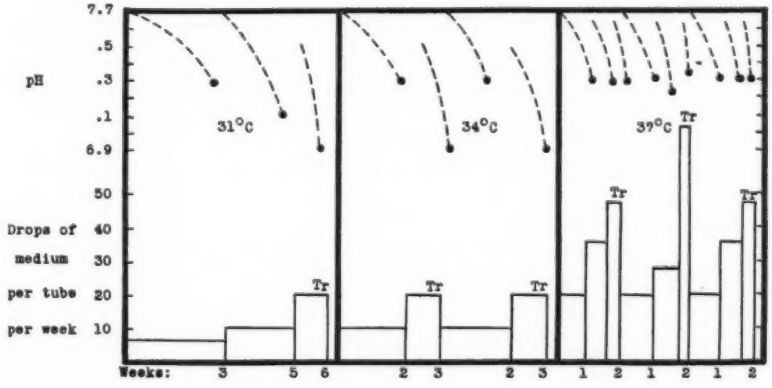



FIGURE 9

THE VOLUMES OF MEDIUM ($S_{40}Em_4$) SUPPLIED PER TUBE PER WEEK, THE RATE OF pH CHANGE AND THE RATE OF TRANSFER OF HUMAN FIBROBLASTS AT 31° C., 34° C., AND 37° C.

Tr=transfer
Total period: six weeks at each temperature

at approximately the same rate in each of three successive series. At 31° C. and 34° C. the successive renewals of medium failed to re-establish the original pH values. Consequently, though the drop was almost exactly 0.5 point on the pH scale, the range fell progressively lower due to an accumulation of acids throughout the plasma medium. It has since been found that the addition of one drop of 1.4% (isotonic) sodium bicarbonate to the ten drops of renewal medium almost exactly corrects the pH of the plasma medium in which the reaction had previously fallen to pH 7.2.

From the data summarized in Table 7, it is apparent that the work

TABLE 7

THE SUPERIORITY OF 31° C. FOR ECONOMICAL MAINTENANCE AND OF 37° C. FOR RAPID PRODUCTION OF NEW GROWTH

| | Maintenance | | | Production of Growth | |
|---|---|---|---|---|---|
| | | | | Requirements for each new Subculture | |
| Temperature | Drops of medium per colony per week | Transfers required in each six weeks | New Subcultures produced per colony | Time (days) | Medium (drops) |
| 31° C. | 1.7 | 1 | 8/42 days | 5.3 | 1.25 |
| 34° C. | 2.2 | 2 | 8/21 days | 2.6 | 0.83 |
| 37° C. | 5.0 | 3 | 12/14 days | 1.2 | 0.83 |

and medium required to carry this strain of cells at 31° C. was approximately one-third of that at 37° C. The growth at 37° C., however, was approximately four times as rapid as at 31° C.

*Role of Concentration of Extract*

Experiments were then undertaken for the purpose of learning the lowest concentrations of embryo extract which would permit continuous maintenance at 31° C. The transferred fragments, three per tube, were imbedded in one drop of chicken plasma, which was coagulated with two drops of $S_{40}Em_2$. After permitting growth for a few days the quality of all colonies was rated on a one to four plus scale and three tubes were assigned to each of five culture groups in which the prospects for further growth were comparable. The liquid phase added to the first group was ten drops of $S_{40}Em_2$, to the second $S_{40}Em_1$, to the third $S_{40}$ alone, while the $S_{40}$ in the fourth and fifth groups was composed of different proportions of fresh and heated serum. Three other experiments were prepared in the same manner.

The general procedure for maintenance in these four experiments differed from that described earlier in two respects: If the medium in some of the tubes in a group had fallen to pH 7.2 more rapidly than those with smaller cultures, such tubes received an interim addition of one drop of sodium bicarbonate; if the pH again fell to 7.2 there was added one drop of sodium bicarbonate and to all of the tubes five drops of the proper liquid medium so that complete replacements of medium could be made only as required by the smaller cultures. In order to continue some of the colonies in the original tubes for as long a period as might be practical and to obtain low temperature material for other experiments, one or sometimes eventually two of the larger colonies were transferred from tubes which utilized the medium more rapidly. This expedient served to confirm periodically the growth potential of transferred colonies and to keep the tubes of each culture group running at more comparable rates. By such means, the original tubes were retained for a period of eleven weeks with four applications of medium, including the initial feeding. In each group of cultures the number of colonies supported per week and the medium required per colony per week was calculated.

Since the average number of drops of medium required per colony per week in the four experiments were closely comparable (1.54, 1.8, 1.5, and 1.47) the total colonies present at each interval and the drops of medium available per week were summarized and averaged (Table 8). Calculation revealed that these cultures were maintained at 31° C. for a period of eleven weeks with an average expenditure of 1.6 drops of medium per colony per week. This was significantly less than the amount (1.7 drop per colony per week) used in an earlier experiment covering only six weeks, since here the large colonies were maintained during an additional period of five weeks in the original tubes.

Another feature of these experiments remains to be considered. There were in all four experiments seventeen tubes which received $S_{40}$ plus $Em_2$ or $Em_1$, and thirty-two tubes lacking embryo juice in the liquid phase. The average medium requirement of the cultures with some embryo juice in the supernatant fluid was 1.7 drops per colony per week, which supports our observation that the size of colonies and the quality of cells were similar to that in the groups with serum supernatant. The embryo juice used in the preparation

TABLE 8

MEDIUM USED TO MAINTAIN HUMAN FIBROBLASTS AT LOWERED TEMPERATURES FOR ELEVEN WEEKS

| Total Colonies Maintained in Four Experiments Using 49 Culture Tubes | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Interval | 0-3 weeks | | 3-5 weeks | | 5-9 weeks | | 9-11 weeks | | | | Total colony weeks |
| Colonies × weeks | 131 × 3 | | 117 × 2 | | 114 × 4 | | 104 × 2 | | | | |
| | 393 | | 234 | | 456 | | 208 | | | | 1291 |
| | Medium Used During Eleven Weeks | | | | | | | | | | Total drops medium |
| Weeks | 0 | 2 | 4 | 6 | 7 | 8 | 9 | 10 | | | |
| Treatment | Orig.* | *r* | *r* | *NaHCO₃* | Add | *r* | *NaHCO₃* | Add | | | |
| Drops per treatment interval | 460 | 460 | 490 | | 130 | 490 | | 25 | | | 2055 |
| Average rate per week | 230 | 230 | 230 | 230 | 123 | 123 | 123 | 253 | 163 | 163 | 188 |
| Average drops of medium per colony per week: | | | | | | | | | | | 1.59 |

*Orig.: Refers to the original medium, *r* to renewal, *NaHCO₃* to addition of sodium bicarbonate, *Add* to additions of the liquid phase.

and the patching of the plasma film in the serum cultures was sufficient, if distributed uniformly throughout the medium, to provide in the serum groups an average level of only 0.2% embryo juice.

Of colonies transplanted during the course of the experiments (and selected as desirable material for new work) nine came from fluids with Em 1 or 2%, while eighteen came from tubes calculated to contain only 0.2% embryo juice. This ratio corresponds closely to the number of colonies available in each group. At the termination of the experiment (eleven weeks), the best colonies were used for further transplantation. Of these, one came from Em 1 or 2%, while nine came from Em 0.2%. The colonies in Em 1 or 2% were viable but more often contained fragments of dead cells as debris. Since cultures from the lowest level of Em concentration were selected for new material more often than might be expected, it is apparent that the dilute serums as fluid phase were highly satisfactory for maintenance at 31° C.

When plasma films are coagulated by means of tissue extract even

in low concentrations, there is always the possibility that the cells derive advantages greater than those which are apparent from calculation of the average concentration of the extract throughout the tube. For this reason, the plasma-coagulating method used in the foregoing experiments cannot be used to learn the lowest levels of embryo juice required for maintenance.

In the next two experiments, the one drop of chicken plasma was hardened by the addition of two drops of fresh human cord serum 50%, and it was proposed to learn whether the cells could be maintained in the complete absence of embryo juice. During the first two weeks, the colonies in $S_{40}$ and $S_{20}$ were regarded as superior to those receiving $S_{40}Em_1$ (control groups). It required in one experiment six weeks and in the other, four weeks, before the culture groups completely lacking embryo juice contained definitely fewer and smaller cells and smaller colonies. After seven weeks, with one interim renewal, transfers were successful, and the new colonies were maintained in both experiments for a further period of ten weeks with only one renewal. The growth rate during this period was less than in the control groups supplied with 1% embryo juice and was just sufficient to replace deteriorating cells. After a total period of seventeen weeks, or approximately four months, in the complete absence of embryo juice, the cells, though fewer, were judged to be of as good quality as those in the control groups. The colonies still contained occasional dividing cells. Whether such colonies can be transferred or maintained for even longer periods remains to be determined.

*Maintenance of Established Colonies at 28° C.*

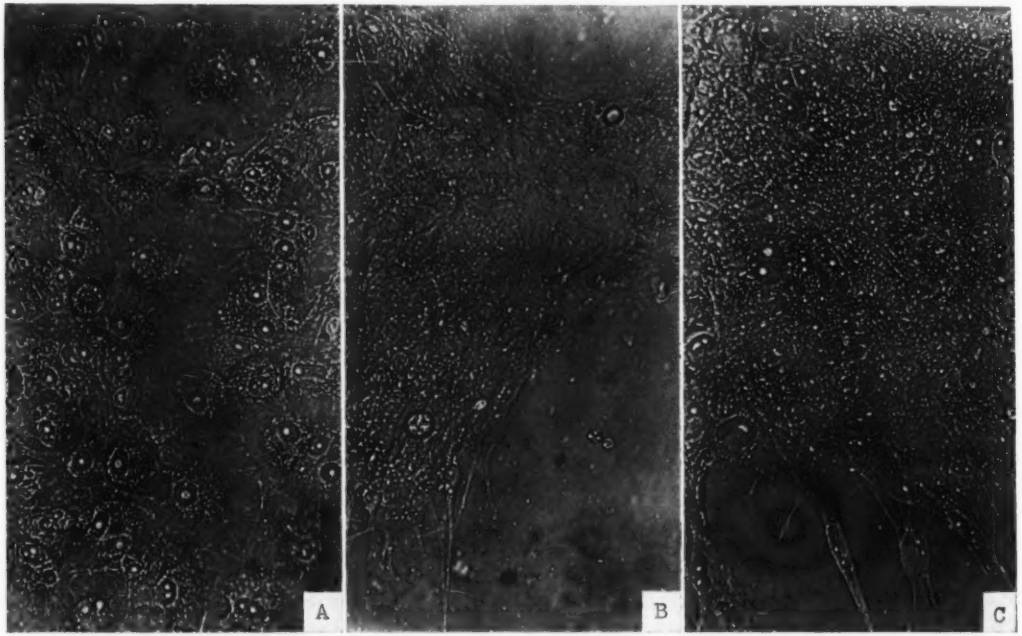
Though continuous transfer of the human fibroblast strain at 28° C. was not studied systematically, sufficient observations were made to suggest that this strain might possibly be carried continuously at this temperature. The storage of small established colonies at this temperature for intervals of four to twelve weeks was a routine practice and always permitted uncomplicated recovery following elevation of the temperature. A group of cultures which had been at 37° C. for one month and contained very large colonies were carried at 28° C. to a total age of six months in the original tubes, the renewals being made only as required. In this interim colonies were removed and transplanted to 37° C. successfully from time to time, and this was still practical when the observations were terminated.

During this storage of large colonies, there was a profound change in the total cell population and in the morphology of the colonies and the cells. The cell population decreased by the loss of the thin peripheral growth which had occurred at higher temperatures. Persistence of the colonies was due to new sprouts which appeared near the central portions of the colony and showed very limited migration among the debris remaining from earlier cells. The cells developed quite frequently into abnormally large stellate forms. In positions where cells grew on the plasma surface or at the interface between the plasma and the glass, they became very thin, flat, and granular. Nevertheless, as holes developed in the plasma from time to time these cells were always ready to push out new normal processes and daughter cells could be found on the glass surface. Whenever these colonies with cells of altered appearance were given a renewal and placed at 37° C., they produced new cells of the usual type within approximately one week. In handling such recovered colonies, it was necessary to separate the new cells from the accumulated debris in order to obtain good cultures. This was most conveniently done by placing a group of six to nine subcultures (those from one colony) in a new tube in a restricted area (bunched subcultures), allowing a short period of growth, and then cutting them again to provide clean cultures for growth as isolated colonies.

## Discussion

In all of the strains studied, with the exception of strain AN-21, the general character and appearance of the cells of each strain differed very little whether the strain was grown at 37° C. or at the low temperatures. The effect which one might expect on the viscosity of the cell when the temperature was lowered did not manifest itself in the general appearance of the cell processes. Cells grown at 28° C. and 31° C. (Figure 9) were as fully expanded as at 37° C., and their processes seemed to be as delicate at the lower temperatures as at 37° C. Aside from the decrease in the rate of migration, fully developed cultures in thin plasma and with a wide range of outgrowth showed a single layer of cells of comparable cytoplasmic density throughout the temperature range.

The nuclei were not especially prominent at the lower temperatures nor was their shape remarkably altered. At the lower temperatures the nucleoli, however, were especially prominent. This was the




37° C. RAR (6) 31° C. RAR (6) 28° C. RAR (6)

FIGURE 9

LIVING SINGLE CELL-LAYER SPREADS OF WALKER RAT SARCOMA 319

Strain over 15 years in continuous culture. (A) Edge of roller tube culture at 37° C. showing one dividing cell. (B) Edge of similar culture at 31° C. showing one dividing cell. (C) Edge of similar culture at 28° C. showing one dividing cell. 31° C. and 28° C. cultures photographed six weeks after transfer with no treatment since transfer. The 37° C. culture ran for three weeks with no treatment, followed by two patches in one week, then a transfer. Photographed at six weeks at which time it shows as seen above slightly swollen and moderately vaculated cells with prominent nucleoli. Six weeks at 28° C. shows slightly granular cells with prominent nucleoli. All cultures show well expanded cells at the periphery of the colony. RAR="Renew as required" schedule of treatment.

most consistent modification of morphology at reduced temperature and could easily be observed at low magnifications. They appeared to be more compact, rounded up, and definitely more refractile.

Although no cells were followed throughout their entire mitotic and inter-mitotic intervals, it was obvious from the data on growth that the mitotic frequency was greatly reduced. More precise information on this aspect of the problem is valuable and additional work is needed.

In some of the cultures (Figure 10) at 31° C. uncommonly large giant cells were found. These included some with several large nuclei and others with many smaller nuclei. Since they persisted for a long time, their ultimate fate was not observed. Their occurrence, how-

FIGURE 10

COMPACT SINGLE-CELL LAYER SPREAD OF *in vitro* PRODUCED RAT SARCOMA STRAIN 333
This sarcoma strain over six years old when photographed. These giant cells appeared to be more numerous at the lower temperatures. Photographed from living 34° C. tube culture.

ever, was very common and may not have been due primarily to the low temperature because in many other cultures maintained under similar conditions they were not observed.

In the media used here and routinely for stock cultures large multinucleated cells or cells with two or three nuclei are fairly common in cultures of many different strains of cells and should not be confused with the excessively large ones referred to above. It has been observed by Gey* from motion picture records that multinucleated cells can divide by mitosis and produce daughter cells with several nuclei. Such cells, therefore, even the excessively large ones, are not necessarily degenerating cells.

In the Strain AN-21 efforts were made to select for low temperature cultivation cultures with cells relatively free of brown granules. Our observations to date do not justify any conclusions regarding the factors contributing to the origin of these granules nor do we know much of their nature. In some of the cultures placed at lowered temperature their appearance was long delayed and successful subcultivation was possible. In other cases their early appearance in abundance was disastrous. We do not believe that they were degeneration granules because continued growth at low temperature was possible. Furthermore, the condition and the growth of these cells at low temperature has been observed to improve when the glucose concentration was reduced. The possibility that they were storage granules was also considered.

The evidence that a relatively acellular tissue such as adult human skin can initiate a cellular outgrowth at 31° C. as reliably as at 37° C. confirms the observations of Hanks (12) on the more active and cellular chick and chick embryo tissues. The early superiority of epithelial outgrowth from human skin as compared to fibroblastic outgrowth is possibly explained in part by the initially greater population of epithelial cells and also by the considerable delay in fibroblast outgrowth at the lower temperature. In view of the temperatures which normally prevail in the skin and the extremities (1, 30) and of the observations of Smith and Fay (31, 32) and Allen (1) on wound healing at lower temperatures, our observations on growth of primary explants occasion no surprise. Under certain circumstances, for example, where cells need opportunity to recuperate from almost

*To be published.

fatal degrees of damage by heat, X-ray or other treatments, the higher energy requirements at body temperature may perhaps reduce their possibilities of survival or recovery. In a study on seven different species of bacteria which had been heated to critical levels, Nelson (21) found 32° C. superior to 42° C., 37° C., 28° C. or 21° C. for the recovery of viable cells. Also, Williams and Reed (34), in studying the effects of heat on Cl. Botulinum, observed a greater proportion of recoveries at 24° C., 27° C., and 31° C. than at 37° C. and with no choice among the lower temperatures.

The use of reduced temperatures to lower metabolic rates and to facilitate the maintenance of stock strains of cells can be based on either of two general procedures. In the first of these, the growth repression is only temporary and is alternated with intervening periods of more rapid growth, in accordance with the biological principle that organisms can withstand retardation for long periods even though they would expire if repressed continuously. This procedure was amply demonstrated in the present study to be practical in the case of those cultures (Strain AN-21 and Walker Rat Sarcoma 319) which, with the media and methods used, could not be propagated continuously at 31° C. or 28° C. The Walker Rat Sarcoma 319 could survive only one serial transfer after approximately 2.5 to 6 weeks at 31° C. or 28° C. before continuation at low temperature became impractical. Even if not renewed at all dividing cells usually persisted for approximately ten weeks and the cultures responded with a delayed wave of mitoses following one renewal after this period. Strain AN-21 survived without renewal for six weeks at 31° C. or for as long as twelve weeks when fed only once each three weeks, provided recovery of normal growth was then allowed to occur at 37° C. As stated previously in the case of Strain 333, cultivation at 28° C. for 15.5 weeks was followed by recovery at 37° C.

Large colonies of the human fibroblasts were stored with occasional renewal for intervals as long as six months at 28° C. and always permitted good recovery at 37° C. This was the principle employed by Fischer in maintaining chick tissue cultures for one month at room temperature, by Hanks in storage of chick cultures at approximately 30° C. for three or four months, and also by Pinkerton and Hass and by Sanders and Molloy in the propagation of infectious agents. Hanks observed a progressive lowering of the

growth potential of chick tissue cultures following each serial transfer at 30° C. of colonies which had been stored for long intervals without renewal. In further examples mentioned above (Strains 319, AN-21, 333), whether due to peculiarities of individual cell strains, the prolonged withholding of nutrient, or the nature of the media employed, the point was reached when renewed stimulation at higher temperatures was necessary. It is obvious from the results discussed that prolonged withholding of nutrient cuts down the probabilities of contamination considerably and constitutes a saving of work and material.

In the procedure applicable to cultures which adapt more readily to continuous growth at reduced rates, this lowering of the growth potential is advantageous, since the "carry-over" of metabolic activity from higher temperatures necessitates for a time a supply of additional nutrient. Among the strains studied, the 14pf, the Emge sarcoma, and the fibroblasts from human skin could be transferred continuously at 31° C. for months and, with a further lapse of time, perhaps indefinitely. The human fibroblasts were maintained throughout the entire period of the study at 31° C., and in dilute serum without embryo juice the growth was successfully brought to a standstill for four months. Incidental observations with colonies transplanted at 28° C. suggested the probability of continuous maintenance at this temperature.

In the absence of perfected means either to freeze or to partially dehydrate living tissue cells, the maximal longevity of renewed cultures at reduced temperatures, like minimal renewal schedules for continuous maintenance, deserves further work. Nemoto's quantitative data from slide cultures serve to emphasize the possibilities in this direction. Our results (Table 5) with Emge Rat Sarcoma at 28° C. show successful maintenance with treatment once in eight weeks and transfer in sixteen weeks, thus requiring only 0.31 drop of medium per colony per week. Nemoto apparently carried chick cells at 30° C. for two and one-half weeks on less than one drop of medium per colony per week (1d/c/wk), while Hanks maintained such cells for more than twelve weeks with 0.8 d/c/wk. Under the differing circumstances described in this paper, the 14pf strain was carried for periods of four weeks with 0.6 d/c/wk, the strain AN-21 for twelve weeks with approximately 1 d/c/wk, and the human fibroblasts for eleven weeks with 1.6 d/c/wk. Since in the latter case,

we were definitely feeding above minimal levels, it would appear that these figures can be surpassed by the use of small fragments, no preliminary incubation of the new cultures at 37° C. or 39° C., for chick cultures and (in the case of cells which permit) the use of stock material which has continuously been at low temperatures.

From the data and experience at hand it is possible to get a tentative view of some of the factors in the medium which influence cell maintenance as the temperature is progressively lowered. At 37° C. continuous growth occurs in $S_{60}Em_6$, $S_{40}Em_4$, and $S_{20}Em_2$, the rate being proportional to the concentration of the medium. At lowered temperatures, we have not had opportunity to investigate systematically the effect of lowering the nutrient content of the medium but have noted several evidences that this might solve the problem for those strains which did not grow continuously at lowered temperature. The Walker Rat Sarcoma 319 was shown to survive longer in serum 25% than in 66%, and some of the difficulties with strain AN-21 were greatly reduced by using one-half the usual concentration of embryo juice and of glucose. Furthermore, in the more concentrated media the longevity of these two strains was found to be greater when the medium was not renewed too frequently.

We now feel that our difficulties with these two strains may have been due primarily to overfeeding. This probability is supported by the interesting observations of Sanders and Molloy on the survival of both guinea pig and chick embryo cells at 23° C. in Simms' serum ultrafiltrate. It is apparent that simple dilution of the complex constituents here used will lower the concentration of all types of molecules, whereas in fact only a few compounds may be present in excess concentration. Other compounds might more usefully be maintained at the levels prevailing in these studies. The small molecules in Simms' serum ultrafiltrate presumably were present in concentrations comparable to those in our media and without objectionable effects. In view of Fischer's work (7), these considerations suggest that the difficulties encountered may have been due chiefly to the larger molecules in our medium and not to the dialyzable components.

The problem of holding cells for long periods of time in a quiescent state, with minimal renewals or subcultures inevitably raises the issue of specific growth requirements and also of the components in the medium which may deteriorate during prolonged incubation.

Hanks had mentioned (12) the possibility of employing non-renewed medium to permit making observations on statistically adequate numbers of colonies containing partially depleted cells as a background in which specific supplements can be made to reveal the difficulties in more prolonged survival. Another alternative is to furnish the usual nutrients at levels giving only minimal growth or survival and in large groups of cultures so prepared to add specific supplements in order that longevity may reveal their usefulness. One of us has more recently emphasized (Gey, Hershey Conference on Tissue Culture) the importance of the analytical approach, which has been so successful in animal, bacterial, and plant nutrition, and in which by bio- and chemical assay one can determine the constituents which become inactivated by incubation or depleted by the cells. We conceive such problems to be most economically studied by methods which do not require constant feeding and continual manipulation of cultures.

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## Summary

1. Some of the conditions necessary for the maintenance of mammalian tissue cultures at reduced growth rates and low metabolic levels were investigated in roller tube cultures of human epithelium and fibroblasts from skin; several strains of autologous normal, altered normal, and malignant rat mesenchyme cells; and two stock strains of rat sarcomas.

2. Reduction of temperature, prolongation of intervals between treatments, and reduction of some of the nutrients (glucose, total proteins) to fairly low levels favor not only prolonged survival at low metabolic levels but also slow continued growth of some cell strains at 28° C. and 31° C.

3. Minimal feeding rates varying from 0.6 to 1.6 drops (36 to 96 cu. mm.) of medium per colony per week sufficed in general to maintain cells at 31° C. Approximately 0.3 drop (18 cu. mm.) per

colony per week at 28° C. permitted maintenance of a rat sarcoma strain with treatment once in 8 weeks and transfer once in 16 weeks. In comparison with one of the usual maintenance schedules for stock cultures with treatment twice per week at 37° C., this minimal feeding rate constitutes better than a fifteen-fold saving of work and medium.

4. The procedures developed in this preliminary study suggest additional means of studying the growth requirements of strains of normal and malignant cells.

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